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PRINCIPAL INVESTIGATOR: James S. Felton, Ph.D.

CONTRACTING ORGANIZATION: Lawrence Livermore National Laboratory
Livermore, California 94550

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James S. Felton, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Lawrence Livermore National Laboratory
Livermore, California 94550

E-Mail: felton1@llnl.gov

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13. ABSTRACT ***(Maximum 200 Words)***

We are investigating the effects of foods associated with reduced prostate cancer risk on a dietary carcinogen known to be associated with cooked meat and elevated cancer risk. Cooked muscle meats contain potent mutagens and carcinogens belonging to the heterocyclic amine class of compounds. One of these, PhIP, is a genotoxic carcinogen that has been shown to cause DNA damage in prostate tissue and prostate tumor formation in rats. We have developed a method to quantify urinary metabolites of PhIP in human volunteers that have been fed a meal of cooked chicken. Using this method, we have shown that PhIP metabolism may be affected by diet and lifestyle factors and that soy may influence the relative amounts of PhIP metabolite excretion. At the cellular level we are investigating the metabolism of PhIP in human prostate cancer cells and have shown that there may be unique metabolic pathways for PhIP and N-OH-PhIP in prostate cancer cells. This research uses state-of-the-art analytical measurement methods to support conclusions about the role of diet and prostate cancer in humans. Although still preliminary, our results indicate that other components of the diet, such as soy and broccoli, may have an effect on the metabolism of a commonly-occurring food carcinogen.

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INTRODUCTION:

This study is designed to determine primary interventions that will prevent PhIP from causing prostate cancer. We are investigating the effects of foods associated with reduced prostate cancer risk on a dietary carcinogen known to be associated with cooked meat and elevated cancer risk. Cooked muscle meats, a prominent component of the Western diet, contain potent mutagens and carcinogens belonging to the heterocyclic amine class of compounds. One of these, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) is a genotoxic carcinogen, causing mutations in bacteria [1] and mammalian cells in culture [2]. There have been several animal studies linking PhIP exposure to DNA damage in prostate tissue or prostate tumor formation [3-5]. In humans, prostate tissue has been shown to activate PhIP, and DNA adducts have been detected in the tissue after metabolic activation [6].

PhIP is naturally formed in meats during the cooking process, with the highest levels found in grilled or fried meats. There are measurable amounts of PhIP in numerous foods, and in very well-done meats, PhIP can be found at levels up to 400 ng per gram of meat [7]. The human intake of PhIP varies with food type and cooking conditions and is estimated to range from nanograms to tens of micrograms per day [8]. We have developed a method to quantify urinary metabolites of PhIP in human volunteers that have been fed a meal of cooked chicken. This method allows us to understand PhIP metabolism in humans and to measure the effects of potentially chemopreventive foods. At the cellular level we are investigating the metabolism of PhIP in human prostate cancer cells as well as the effect of several of the putative active ingredients in the potentially chemopreventive foods.

Progress during Year 2:
TASK 1: Determine the stability of PhIP metabolism

A) Determine the stability of PhIP metabolism within an individual over time. Three healthy, normal, male volunteers have been recruited to participate in this phase of the study, which will continue during Year 3. Subjects are asked to abstain from meat consumption for 24 hours prior to being fed a meal that contains 150 g cooked chicken with a known PhIP content. Control urine is collected before the chicken meal and for four 6-hour periods (24 hours total) after eating the chicken. Participants are asked to further abstain from cooked meat during the urine collection period. No other dietary restraints are placed upon the individuals. Urine samples are analyzed and metabolites are measured as described in Kulp et.al [9]. We quantify four major human PhIP metabolites: N^2-OH-PhIP-N^2-glucuronide, PhIP-N^2-glucuronide, 4'-PhIP-sulfate, and N^2-OH-PhIP-N^3-glucuronide in the urine for each sample.

At this time, 2 of the volunteers have consumed chicken and collected urine 7 times and one of the volunteers has participated 6 times. Table 1 shows the subjects and

times when each subject participated. For some of the trials, the urine has been collected and stored, but has not yet been analyzed (no data).

Table 1. Subject participation in metabolism stability project.

Subject A	Subject B	Subject C
12/99	12/99	
4/00	4/00	4/00
8/00	8/00	8/00
12/00	12/00	12/00
6/01	6/01	6/01
10/01 (no data)	10/01 (no data)	10/01
2/02 (no data)	2/02 (no data)	2/02 (no data)

The preliminary results from these feeding trials are presented in figures 1 to 3 and Tables 2 and 3. Figure 1 shows the percent of the total PhIP dose recovered as PhIP metabolites in the urine. The bars are divided into segments representing the contribution of each individual metabolite. In all subjects and in all trials N^2-OH-PhIP-N^2-glucuronide is the major PhIP metabolite, followed by PhIP-N2-glucuronide.

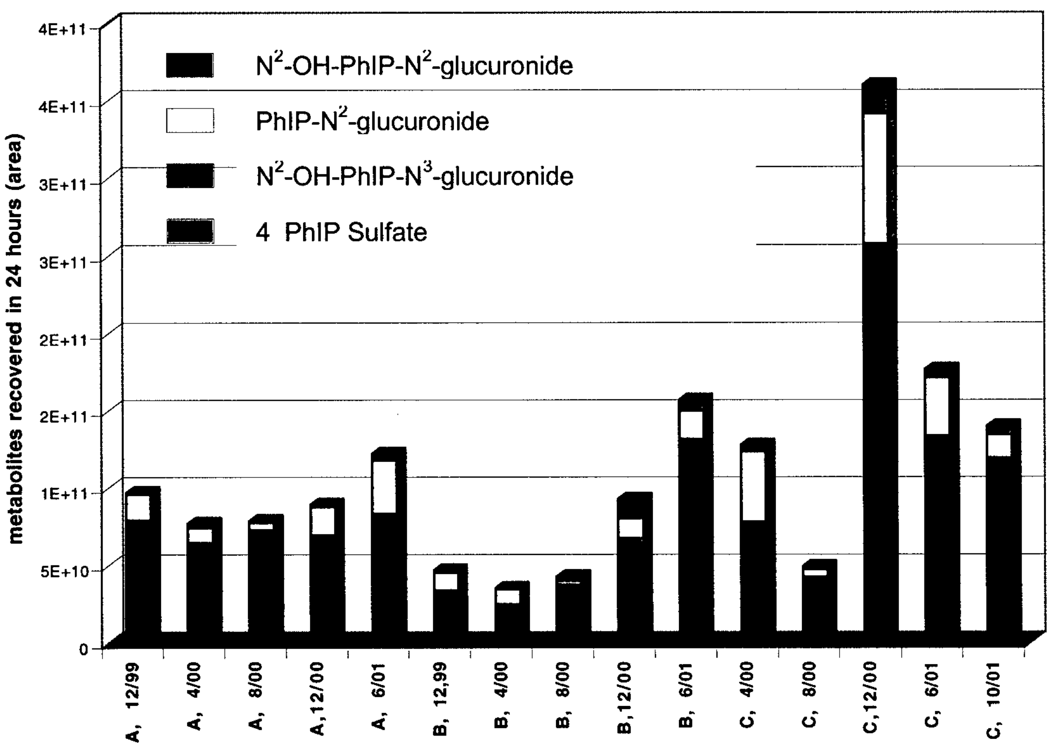

Figure 1. Excretion of PhIP metabolites. Data represent the fraction of the total PhIP dose consumed in the chicken that is recovered as urinary metabolites. Bars are divided according to the fraction that each metabolite represents of the recovered dose.

Together these 2 metabolites account for 70-96% of the excreted metabolite. The ratio of metabolites varies both among the individual volunteers and within the same volunteer over time.

Because we collect urine in four 6-hour aliquots, we are able to determine the rate of metabolite excretion (Figure 2). Typically, most of the metabolites are excreted in the first 12 hours after consuming the chicken, although the pattern of metabolite excretion is not consistent within the subjects over time.

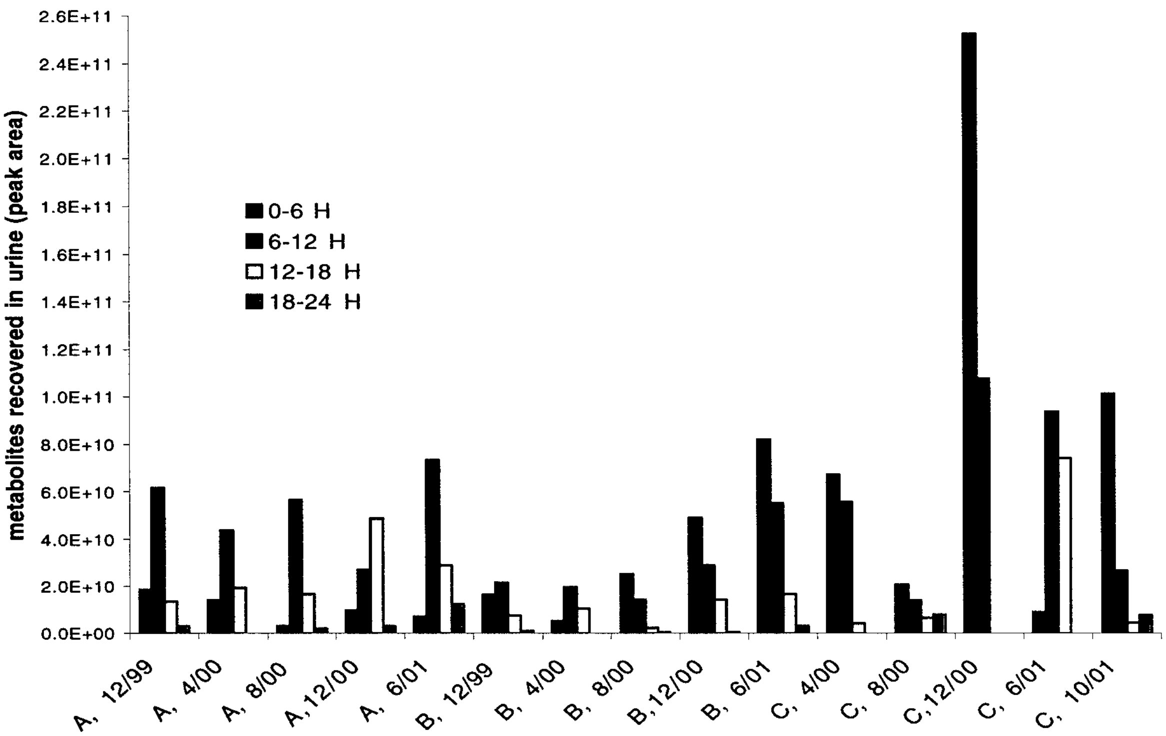

Figure 2. Rate of PhIP metabolite excretion. Each bar represents the total of all metabolites excreted during the given time period as peak area.

If the results of the data for the five trials are averaged for each subject (Table 2), it appears that Subject A tends to excrete more metabolites during the later time points, compared to subjects B & C, who tend to excrete more quickly.

Table 2. Average excretion pattern for 3 subjects.

	Subject A	Subject B	Subject C
0-6 H	11.7 +/- 6.9	42.9 +/- 18.1	48.5 +/- 27.3
6-12 H	56.6 +/- 15.7	40.4 +/- 10.1	34.8 +/- 13.5
12-18 H	27.8 +/- 15.7	15.7 +/- 9.1	12.4 +/- 17.3
18-24 H	3.9 +/- 3.8	1.1 +/- 0.9	4.3 +/- 7.0

We have also calculated the amount of the PhIP dose given in the chicken that is recoverable in the urine as metabolites. Recoveries range from 3-72%, although most

recoveries are less than 25%. The average recovery for all subjects in all trials is 19%. Differences in the PhIP metabolites recovered in the urine may reflect individual variation in digestion and absorption and variation in uptake due to binding of PhIP to other gastrointestinal contents. We are currently pursuing studies to determine how much of the PhIP present in the meat becomes bioavailable in the digestive tract using an *in vitro* digestion system. In these experiments, approximately 23 % of the PhIP present in the chicken becomes bioavailable during the digestion procedure. This suggests that most of the ingested dose that we cannot account for remains undigested and is excreted in the feces.

Figure 3 shows the relationship between the amount of PhIP given in the chicken and the excretion of N^2-OH-PhIP-N^2-glucuronide for all of the volunteers that have participated in the study to date.

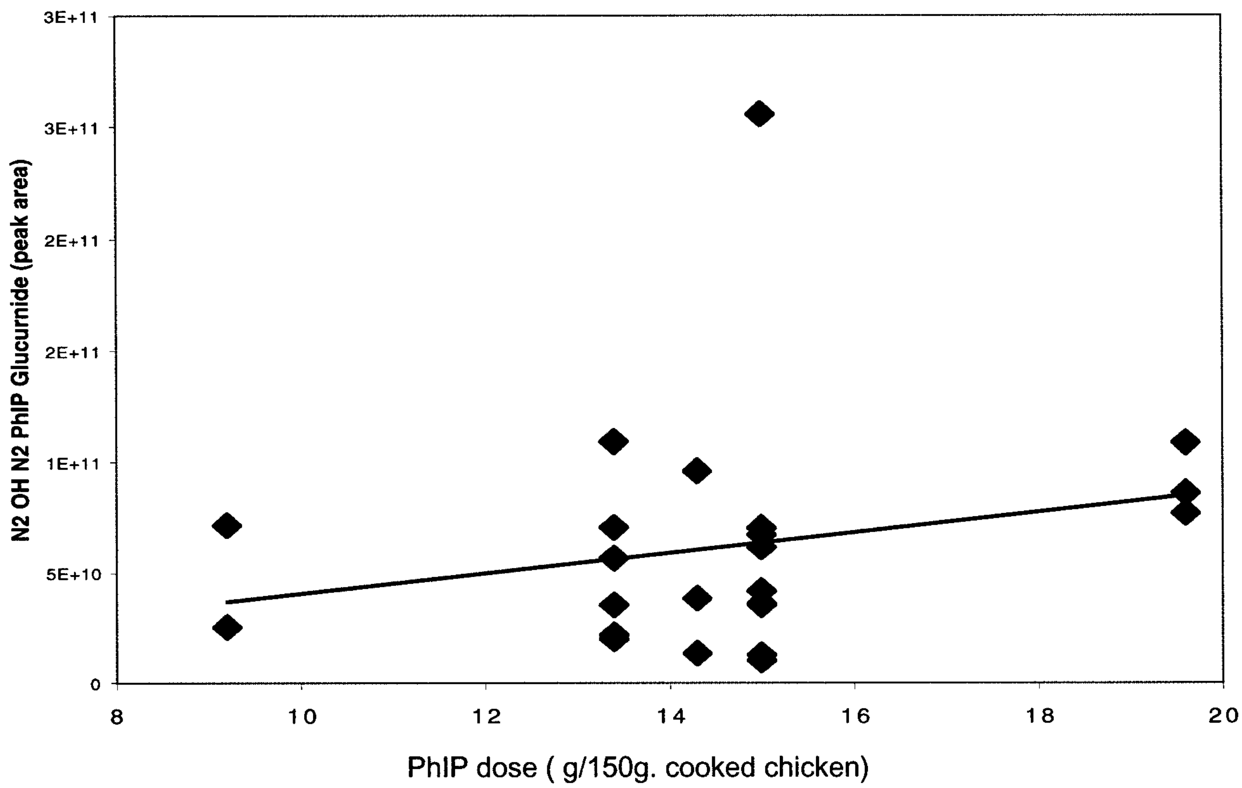

Figure 3. The relationship between PhIP dose and the amount of metabolite excreted in the urine. Data are for all volunteers participating to date.

The amount of metabolite excreted tends to increase as more PhIP is given to the volunteers, but there is not a strong statistical correlation between the 2 factors. A recently published study of PhIP metabolite excretion in humans demonstrated a stronger correlation between excretion of this metabolite (measured indirectly as a 2 OH-PhIP, a breakdown product of N^2-OH-PhIP-N^2-glucuronide) and PhIP dose [10],

although the data contain the same type of scatter seen in our study. It is possible that as we add more data, our correlation will become stronger.

We can also examine the total amount of PhIP metabolite excreted for each of the volunteers for each feeding trial (table 3). It appears that, regardless of the dose given, Subject A consistently excretes an average of 2.8 g of PhIP metabolites. The other 2 subjects demonstrate more variability in the amount of metabolites excreted. It is possible that there are individual differences in physiology that determine the amount of PhIP absorbed or excreted. Human intestinal cells contain active transport proteins that have been shown to play a role in PhIP absorption [11]. These may have an effect on the amount of PhIP absorbed for each individual.

Table 3. PhIP metabolites recovered (g) for each volunteer in each of the feeding trials. PhIP dose (g/150 g. cooked chicken) are given in parentheses.

	Subject A	Subject B	Subject C
	2.9 (9.2)	1.4 (9.2)	3.9 (13.4)
	2.3 (13.4)	1.1 (13.4)	1.5 (15.0)
	2.4 (15.0)	1.3 (15.0)	10.9 (15.0)
	2.7 (15.0)	2.8 (15.0)	5.4 (19.6)
	3.7 (19.6)	4.7 (19.6)	4.2 (14.3)
Average	2.8	2.3	5.2
Std. Dev.	0.6	1.5	3.5

Analysis of the preliminary data presented here would indicate that there is little consistency in PhIP metabolism within an individual over time and that there is more to be understood about PhIP absorption and metabolism. LC/MS/MS analysis of many of these samples will be repeated and the study is ongoing, so no final conclusions can be made. The enzymes known to be involved in the metabolism of PhIP are found at a variety of levels and activities within the human population [12]. In addition, the activities of these enzymes are changeable and can be affected by diet and lifestyle. Variation in the amounts of PhIP metabolites excreted suggests variation in activity levels of the metabolizing enzymes.

Task 1, B) Determine the assay variability of the same urine sample.

This task began during the last 6 months of the first year and will extend into year 3. We are doing repeated analysis of one urine sample to determine the stability of the metabolites over time (in urine frozen at -20°C) and the reproducibility of the LC/MS/MS method. The results of several assays of one urine sample are presented in Table 4.

Sample variability continues to be an issue for the urine analysis. Variation in single samples averages 40% over time. There are two factors that contribute to the problem. The first is the interference from the complex urine matrix itself. The other factor is the

response of the LC/MS. Quantitation is a chronic problem with LC/MS analyses due to changes in the ion path over time. We have addressed these problems by diverting the sample flow from the mass spectrometer during the HPLC equilibration and the first 10 minutes of each run. This keeps a substantial part of the contaminants out of the instrument. Secondly we are now more aware of the contamination of the capillary heater in the mass spectrometer itself. Although no change in system vacuum can be detected with a dirty capillary heater, a degradation in instrument response can be restored with cleaning in 20% nitric acid, which will be done along with calibration to help insure consistent instrument response over time.

Table 4. Assay variation for one urine sample. Numbers represent peak area. Each peak area is the average of three injections.

Date Analyzed	N^2-OH-PhIP-N^3-glucuronide	N^2-OH-PhIP-N^2-glucuronide	PhIP-N^2-glucuronide	4'-PhIP-sulfate
15-Sep-00	9127608	43635865	5261920	3885015
09-Nov-00	4044227	103973710	3273079	3795911
03- Apr-01	14460631	73286162	5535854	5505567
04-Apr-01	6108774	66487416	5465155	2921107
12-Jul-01	11369507	47466902	13732126	2249085
06-Sep-01	5324002	75174655	16202005	3401429
12-Sep-01	7640448	43154840	18332526	5569420
03-May-01	7534177	37273348	8654863	1676218

To address the complex urine matrix issue, we have tried to modify the urine extraction procedure to minimize external interferences, while maximizing metabolite recovery. We have tried new solid phase extraction columns, including Certify", Oasis MCX, and Abselut", as well as adding new washing steps throughout the procedure. Figure 4 compares chromatograms of the same urine sample extracted with 2 different SPE columns. Mass chromatograms in set A show the results of our typical extraction procedure using an Oasis HLB as the first column step. Part B shows the mass chromatograms for the analysis of the same sample extracted with an Abselut" column as the first step and with the addition of a washing step. Although the recovered areas of the two chromatogram sets are not significantly different, set B shows sharper peaks with less interference. We are now switching to Abselut columns for our routine extractions, hoping that that will lessen some of the sample variability.

We have also changed the protocol to add an internal standard after sample extraction as a check on autosampler reproducibility and retention times. These data will be useful to determine sources of variation and be used to improve reproducibility. It is our goal to not have to inject each sample three times to obtain consistent results.

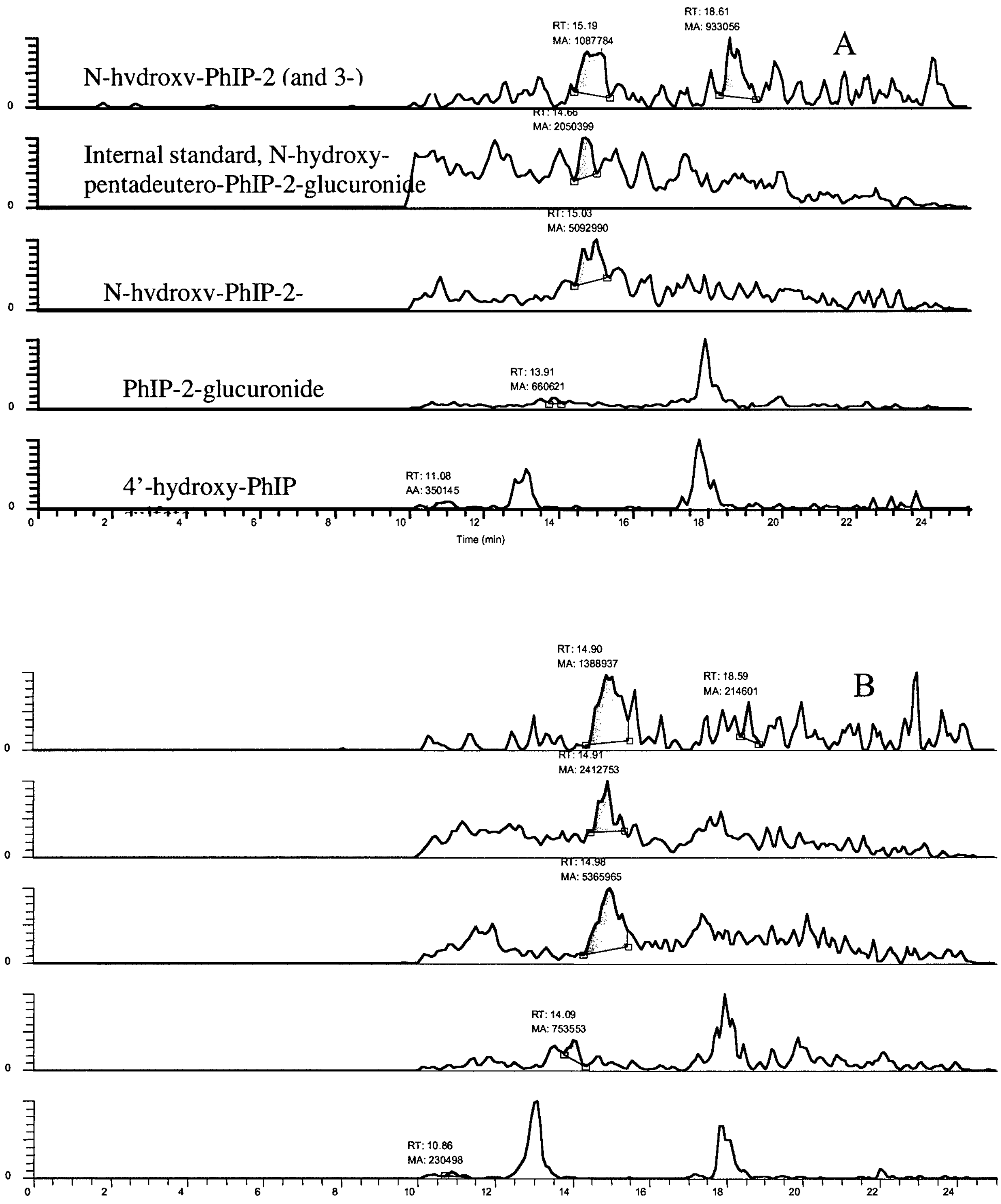

Figure 4. The same urine sample extracted with and Oasis brand SPE column (A) or Abselut brand column, (B).

We are also trying to improve the urine analysis method by the addition of another internal standard, deuterium-labeled PhIP-N^2-glucuronide. For this metabolite there is frequently a high background making quantitation difficult and too heavy of a reliance on the metabolite retention time. A heavy isotope-labeled internal standard will be a check on the retention time and the yield through the extraction process. Since this is one of the major metabolites, accurate quantitation will greatly improve the overall method. We have attempted to reproduce a literature report of the enzymatic synthesis of PhIP-N2-glucuronide from PhIP using the human UGT 1A1 enzyme in the presence of alamethicin [13]. The initial reaction only gave very low amounts of product and the reason for the low yield is not clear. Another sample of the commercially available UGT 1A1 enzyme had been ordered and a new synthesis will be attempted soon.

TASK 2: Human Prostate Cells in culture
A) Effects of PhIP, N-OH-PhIP and 4'-OH-PhIP on cell proliferation

The effects of PhIP and a Phase I metabolism intermediate, N-OH-PhIP on cell growth in the prostate cancer cell lines LNCaP and PC3 was completed in Year 1. The effects of the other Phase I metabolism intermediate, 4'-OH-PhIP, have not been measured at this time. 4'-OH-PhIP is not available commercially and has proven to be more difficult to acquire than we expected. We hope to receive this compound very soon from a collaborator, and will complete those tasks requiring 4 -OH-PhIP when we have the compound in hand.

Task 2, B) Macromolecular binding

This task was begun in Year 2 and will continue into Year 3. PC3 and LNCaP cells were exposed to [3H] PhIP and [3H] NOH-PhIP for 2 hours in a 37ßC incubator. The cells were then removed from the plates by scraping and centrifuged at 700 x g for 10 minutes. The media was removed from the cell pellet and stored frozen at -80 ßC for metabolite analysis. The cell pellet was resuspended in 2 volumes ice cold PBS and split into 2 fractions. The fraction designated for protein analysis was centrifuged and resuspended in ice cold MeOH and stored prior to covalent binding analysis. The remaining fraction was washed again in cold PBS and stored for DNA analysis. No further work has been done on this fraction at this time.

Covalent Binding Studies: Precipitated protein samples were removed from the freezer and centrifuged at 2000rpm for 10 min. The supernatant was removed and the pellet was washed with MeOH until no further radioactivity could be detected in the wash. The pellet was washed with acidic MeOH to remove any unbound compound, washed with ethanol and solubilized in 1N NaOH at 60 ßC. Aftersolubilization, the samples were diluted with water and neutralized with 3 N HCl. They were then mixed with scintillation cocktail and held in the dark for 24 hours prior to counting. Radioactivity was assessed using external-quench method and standardized to protein content. The results of the first experiment are shown in Table 5.

Table 5. Covalent binding of [3H] PhIP and [3H] NOH –PhIP in LNCaP and PC3 cells. Results are DPM/unit protein

	LNCaP	PC3
PhIP	21.7	71.1
NOH-PhIP	124	22.7

It appears that there is differential ability of the 2 cell lines to bioactivate PhIP and NOH-PhIP. PC3 cells, which are not androgen responsive, activate more of the parent compound to a form that is able to bind protein. This may indicate active Phase I enzyme systems in these cells. LNCaP cells, which are androgen responsive, are more able to activate NOH-PhIP. This may due to active Phase II enzyme systems. More work will be done in year 3 to understand these preliminary data.

Task 2, C) Prostate cell metabolism

During Year 1, prostate cell metabolism of PhIP and N-OH-PhIP was assessed by adding these compounds to cell medium for times up to 48 hours, followed by

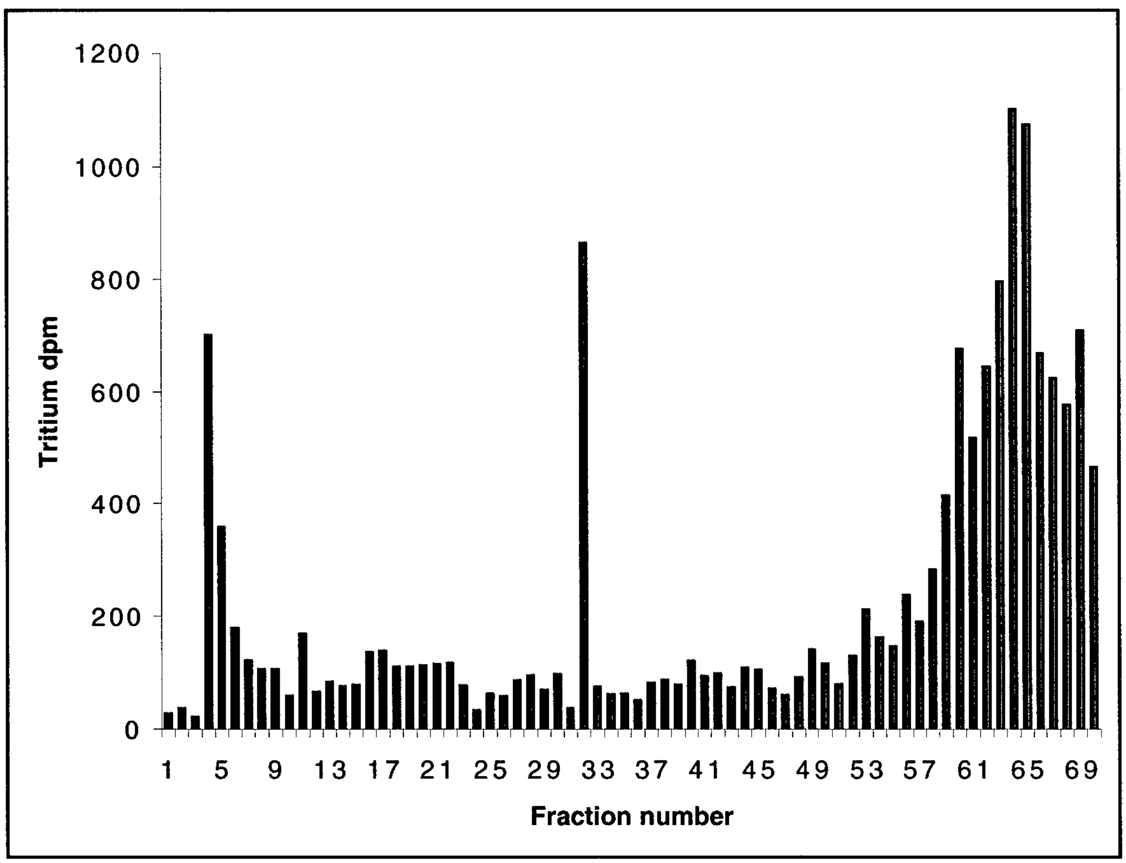

Figure 5. Plot of the HPLC separation of the medium of LNCaP cells treated with PhIP.

LC/MS/MS analysis of the cell medium. Using this method, we tentatively identified one PhIP-glucuronide and N^2-OH-PhIP-N^2-glucuronide, the largest urinary metabolite,

as possible metabolites of PC3 cells. No metabolites were identified in the LNCaP cells using this method. Confirmation of the identity of the unknown PhIP-glucuronide proved difficult because of the small amounts produced by the cells. To further investigate the metabolism of these cells we analyzed the medium collected after incubation of the cells with [3H] PhIP and [3H] NOH-PhIP described above. The only sample preparation was evaporation to dryness so metabolites would not be lost during processing. Samples were injected into the HPLC and one minute fractions were collected and counted in a liquid scintillation counter.

A plot of the radioactivity from the medium from LNCaP cells treated with tritium-labeled PhIP is shown in Figure 5. PhIP has a retention time of 65 minutes under these conditions, so the radioactivity seen at other retention times indicates some metabolism. The large peak at fraction 32 does not co-elute with known metabolites and its identification will be investigated.

With PhIP, the PC3 cell line also shows unretained radioactivity in fractions 4 and 5, and also radioactivity in the 50 to 60 min fractions suggesting metabolites with poor chromatographic behavior.

With NOH PhIP, the LnCaP and PC3 cell lines show the polar metabolites in fractions 4 and 5 but no other distinctive peaks about the radioactivity background. These experiments will be repeated and results further investigated.

It is apparent from these investigations that prostate cells do not produce the same metabolites that we identify in the urine, suggesting that these cells may have unique pathways.

We have considered several possible ways to increase our ability to detect prostate cell metabolites- adding more substrate, increasing the cellular mass, increasing the incubation time and inducing the relevant enzymes so that more metabolites are produced. In an effort to induce Phase II metabolizing pathways, we treated PC3 and LNCaP cells with chrysin, a dietary flavanoid that has been shown to increase glucuronidation and inhibit PhIP binding in Caco-2 intestinal carcinoma cells [14, 15]. Our hypothesis was that treating the prostate cells with this compound would induce glucuronosyl transferases, thereby increasing metabolite amounts.

To establish the feasibility of this approach, we did cell proliferation assays to determine if treating prostate cells with chrysin would prevent the toxic effects of N-OH-PhIP. Because glucuonidation is presumed to be a detoxification pathway, inducing these enzymes should increase the efficiency of N-OH-PhIP metabolism, decreasing the amount of cell death. The results of this experiment are shown in Figure 6. Cell proliferation is assayed with the CellTiter 96 Nonradioactive Cell Proliferation Kit (Pro-Mega) that measures cellular conversion of a tetrazolium salt into a blue formazan product. Cells are plated in 96-well plates and the absorbance of each well is determined spectrophotometrically at 595 nm. Absorbance read is directly proportional to cell number. MCF-7 breast cancer cells, which are also derived

from hormonally responsive tissue, are included for comparison purposes. Treating the prostate cancer cell lines with 1 g/ml N-OH- PhIP for 24 hours decreases cell number by 15-20%. In contrast, the MCF-7 breast cancer cell line is not affected by N-OH-PhIP at the same concentration, leading us to speculate that 1) the toxic intermediate causing the cell death is not produced in these cells or 2) the cells have a mechanism for detoxification not found in the other cell lines. Treating cells with increasing amounts of chrsyin also causes some cell death in the PC3 cell line but has little or no effect on the LNCaP and MCF-7 cells. When the cells are treated with both chrysin and NOH-PhIP, the toxic effects of the NOH-PhIP are potentiated in the prostate cell lines, contrary to our original hypothesis that this compound would be protective. Interestingly, chrysin does not have the same effect in the MCF-7 cells. This suggests that chrysin may induce a pathway that causes NOH-PhIP toxicity that is present in the prostate cells and not in the MCF-7 cells or Caco-2 intestinal carcinoma cells.

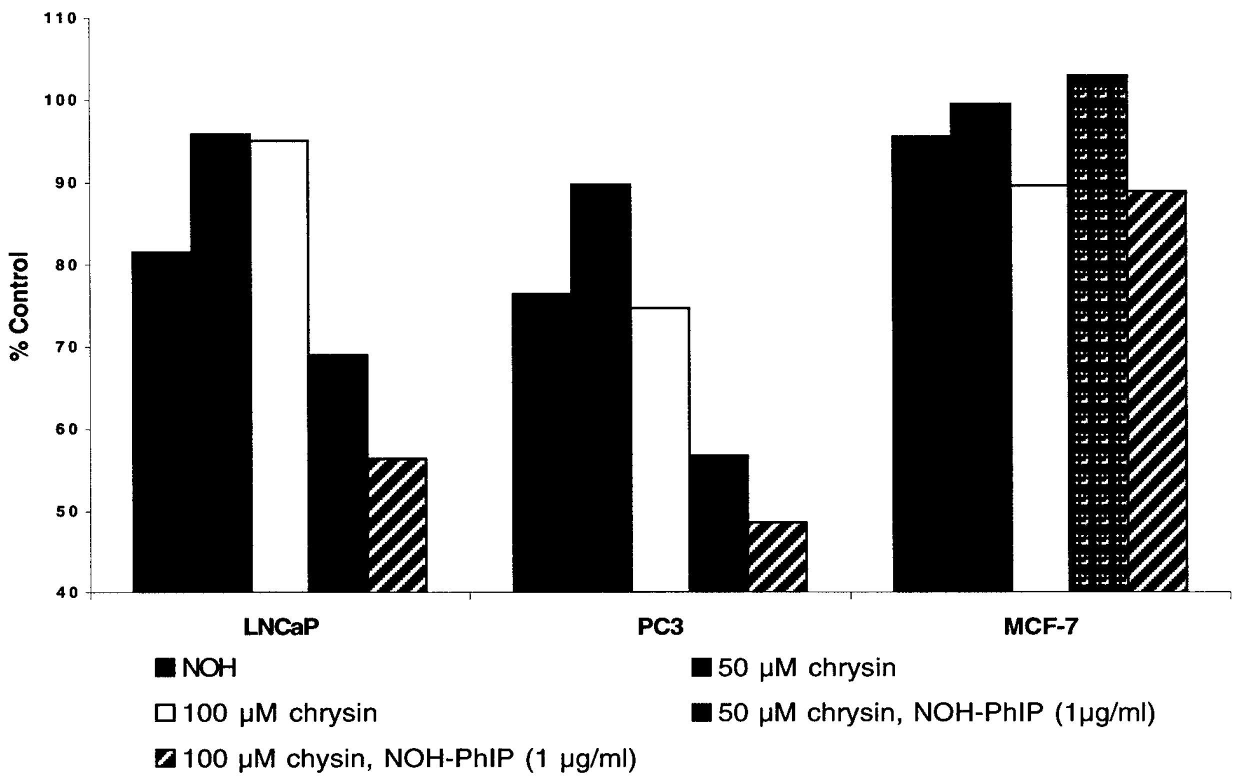

Figure 6. Effect of chrysin on NOH-PhIP (1 g/ml) treatment of breast and prostate cells.

Although these data do not support our original hypothesis that chrysin will increase PhIP metabolite production, the results from this experiment may eventually lead to new understanding about the toxicity of NOH-PhIP in prostate cells. These results may be used as preliminary data to obtain further funding to investigate the effect of PhIP exposure on the prostate.

TASK 3: Link cellular metabolite profiles to urinary metabolite profiles

This task will be accomplished after more is known about the metabolites produced by the cells and we have more results from the macromolecular binding experiments. At this time, we have only found one of the PhIP metabolites in both the cells and the urine. This task will be accomplished in year 3.

TASK 4: Chemopreventive interventions

Although not scheduled until year 3, progress has been made on experiments to investigate the effects of chemopreventive interventions on PhIP metabolism. We have recruited four men to participate in the tomato/lycopene intervention, eight men in the soy intervention and seven men in the broccoli study. More volunteers will be recruited in Year 3 and analysis of the urine samples will continue throughout the final year of the grant.

To investigate the effect of the intervention food on PhIP metabolism we quantify changes in PhIP urinary metabolites. In these studies, we fed the volunteers well-cooked chicken, collected urine and measured a baseline PhIP urinary metabolite profile. We then gave the subjects the intervention food daily for 3 days. On the fourth day we fed them chicken again and collected urine for another 24 hour period.

A) Effect of lycopene in tomatoes on PhIP metabolism in humans and in prostate cells

We have recruited 4 volunteers to participate in this study to date. The intervention food for this study was 1/2 c. commercially available pasta sauce daily at lunch for three days.

To provide the human volunteers with a higher dose of lycopene that is still representative of a typical diet, we examined the literature to find the best food source. Cooked tomato products have the most lycopene. We analyzed three tomato products using a spectrophotometric assay published by Rao et al. in 1998 and Arias et al in 2000 [16, 17]. Three different samples of spaghetti sauce, Ragu Chunky Garden, Ragu traditional, and Prego Roasted Pepper were analyzed along with a negative control of a marinade sauce that contained no tomato products. These were extracted using hexane/acetone/methanol and the absorbance of the organic layer read in a spectrphotometer at 502 nm. All three tomato-containing sauces had lycopene, but not the marinade negative control. The Ragu Traditional sauce contained the most lycopene, about 30% more than the Ragu Chunky Garden and about five times more than the Prego Roasted Pepper. Thus the Ragu Chunky sauce was fed to the volunteers,

Urine from the intervention study has been collected but not yet analyzed.

The effect of lycopene in cells will also be accomplished in Year 3. Lycopene is unstable in aqueous cell medium (half-life less than 2 hours), and it has been suggested that solubilization in micelles provides a more stable delivery system [18]. We are currently working out the method for micellular formation for treating the prostate cells.

B) Effect of soy on PhIP metabolism in humans and genistein in prostate cells

We have begun analyzing the urine from the seven volunteers who have participated in the soy intervention. In this trial the intervention food was a soy shake which contained 8 ounces of soy milk, 1 TBSP of a commercially available soy powder, bananas and honey. The shake was provided to the volunteers daily for 3 days. Although analysis of the samples is on-going and data interpretation may change, it appears that there is a trend toward an increase in N-hyrdoxylation of the PhIP metabolites after soy consumption. Figure 7 shows the total excretion of the 2 PhIP NOH-glucuronide isomers, expressed as percent of the total of all metabolites excreted.

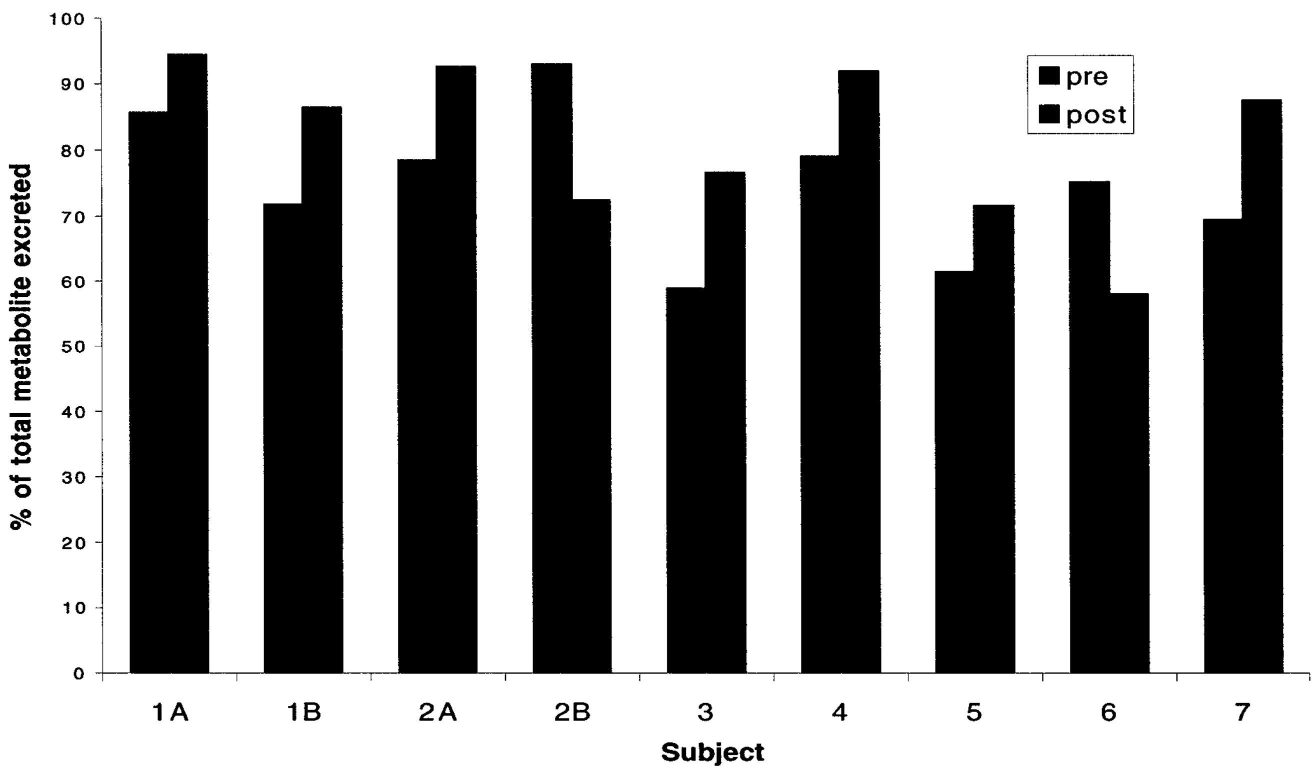

Figure 7. Excretion of the 2 NOH-PhIP-glucuronide isomers, expressed as the percent of the total metabolite excretion, pre- and post soy consumption. Subjects 1 and 2 participated in the study twice.

With the exception of the second trial of Subject 2 and Subject 6, the fraction of the metabolites excreted that were N-hyrdoxylated increased in all of the subjects. Soy milk and soy powder are complex mixtures that contain a variety of biologically

active substances; it is possible that one or several of the components in this mixture induce P4501A1, the enzyme responsible for N-hydroxylation of PhIP.

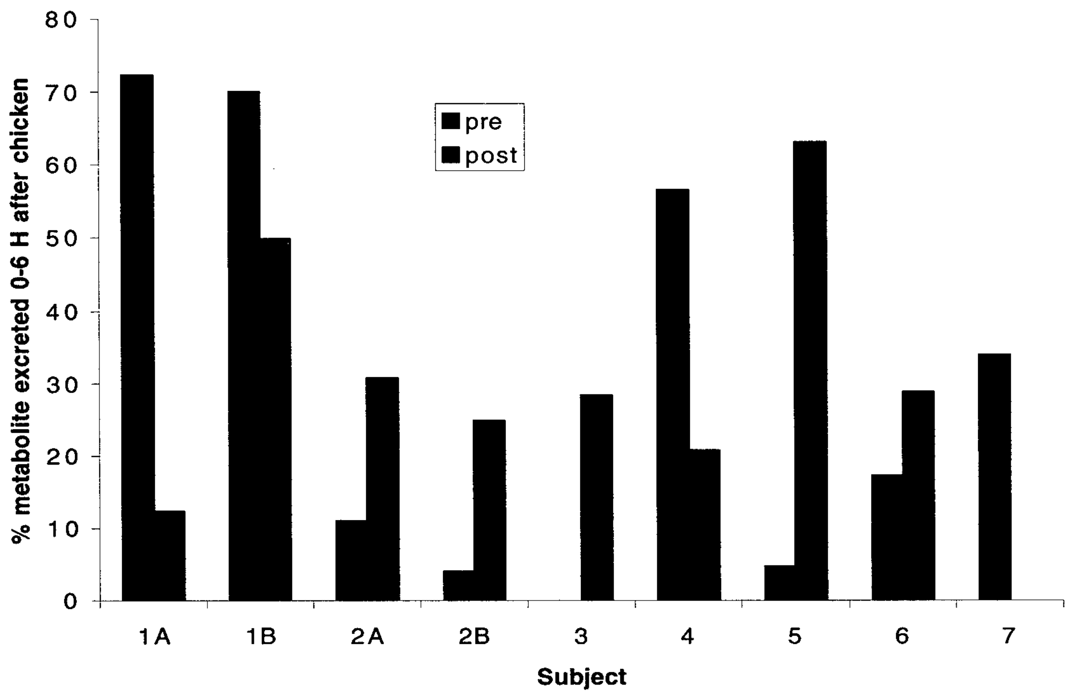

Figure 8. Excretion rate of PhIP metabolites, before and after the soy intervention.

In contrast to the broccoli intervention, which showed an effect on the rate of PhIP metabolite excretion (reported in Year 1), soy does not seem to change the speed of metabolism in the subjects in any consistent pattern. Figure 8 compares the percent of the metabolites excreted in the first six hours after chicken consumption before and after soy consumption. There are no clear trends apparent among the volunteers after soy consumption.

Recent literature reports have shown that genistein inhibits prostate cell proliferation at doses greater than 10 M at greater than 72 hours incubation [19-22]. Because we are giving our volunteers lower doses of genistein and for relatively short time periods, we measured the effect of lower dose of genistein treatment on LNCaP and PC3 cell proliferation for 24 and 48 hours respectively (figure 9).

In these preliminary data (which will be repeated), short time incubations of low doses of genistein cause only a small effect on prostate cell proliferation and the response does not appear dose-dependent. These results will be confirmed during Year 3.

We also investigated the ability of genistein to protect prostate cells from NOH-PhIP -induced cytotoxicity. These results are presented in Figure 10. In this experiment,

LNCaP and PC3 cells were treated with NOH-PhIP alone, genistein alone or a combination of the 2 compounds for 24 hours. The results suggest that although low

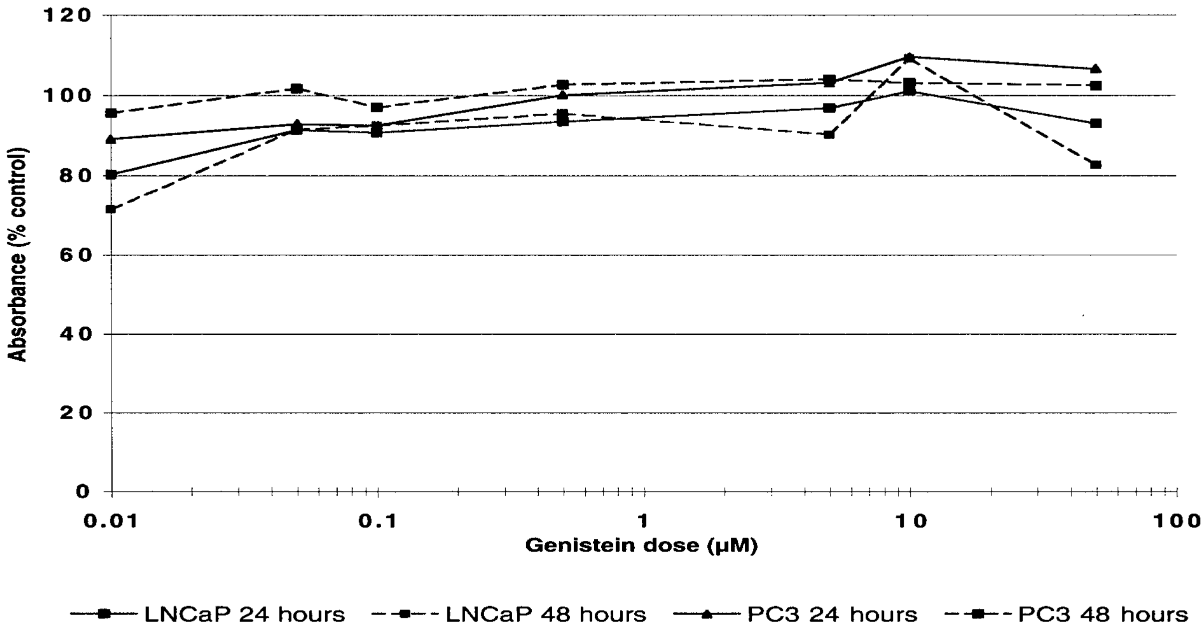

Figure 9. Effect of genistein on cell proliferation in LNCaP and PC3 cells.

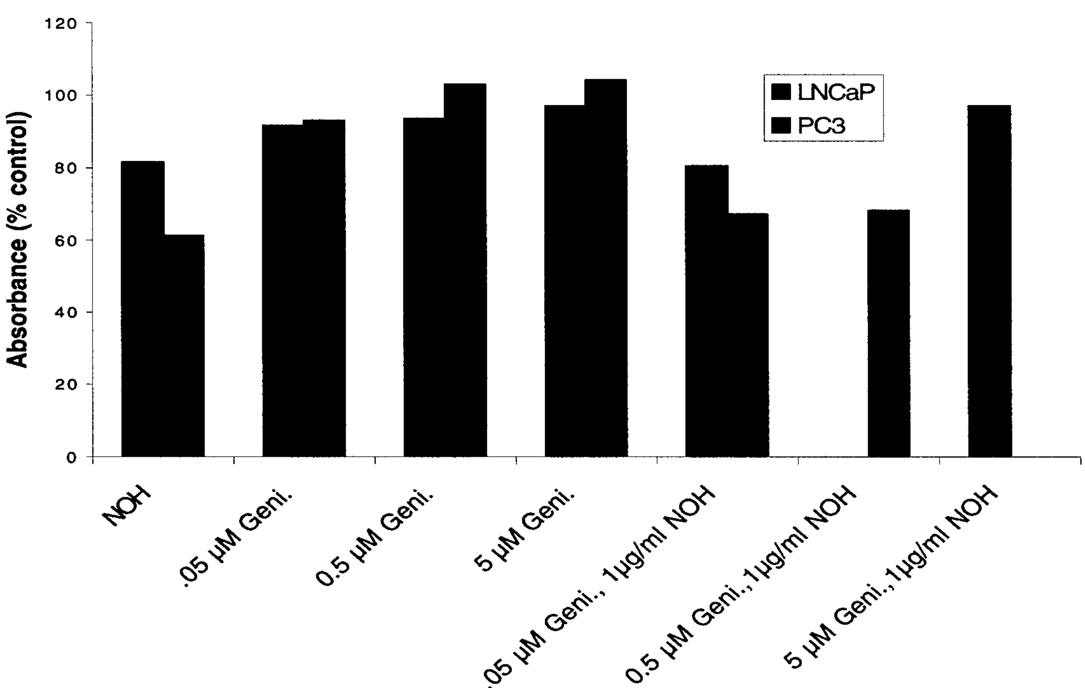

Figure 10. Effect of NOH-PhIP and genistein, alone or in combination, on the growth of LNCaP and PC3 prostate cancer cells. Geni.= genistein, NOH= N-OH-PhIP

doses of genistein do not protect the cells from NOH-PhIP cyctotoxicity, higher doses may. This experiment will be repeated and expanded during Year 3.

C) Effect of broccoli on PhIP metabolism in humans and sulforaphane in prostate cells

The progress on this task was discussed in the progress report for Year 1. No further work has been done on this task at this time.

KEY RESEARCH ACCOMPLISHMENTS:

- Determined that PhIP metabolism is not stable over time and may be highly dependent upon diet and lifestyle factors.
- Improved the sample preparation procedure to lower the impact of interfering substances in the urine and decrease the variation of LC/MS analysis.
- Determined that prostate cell metabolites differ from metabolites that we quantify in the urine.
- Determined that chrysin, a dietary flavonoid potentiates the cyctotoxicity of prostate cancer cells, but not other cell types.
- Determined that soy consumption may affect the relative amounts of PhIP metabolites excreted.

REPORTABLE OUTCOMES:

Manuscripts:

M.G. Knize, K.S. Kulp, C.P. Salmon, G.A. Keating and J.S. Felton, Factors affecting the human heterocyclic amine intake and the metabolism of PhIP . *Mutation Research*, in press.

J.S. Felton, M.G. Knize, C.P. Salmon, M.A. Malfatti, and K.S. Kulp. (2002) Human Exposure to Heterocyclic amine Food Mutagens/ Carcinogens: Relevance to Breast Cancer . *Environmental and Molecular Mutagenesis,* 39:112-118

Knize, M.G., Kulp, K.S., Malfatti, M.A., Salmon, C.P., and Felton, J.S. (2001)"An LC/MS/MS urine analysis method to determine human variation in carcinogen metabolism". *Journal of Chromatography A*, 914:95-103.

Posters and Presentations:

K.S. Kulp, M.G. Knize, S.L. McCutchen-Maloney, and J.S. Felton, PhIP metabolites in human urine and human cancer cells: Implications for individual variation in carcinogen metabolism and chemoprevention American Association of Cancer Research, San Francisco, CA, April 6-10, 2002

J.S. Felton, K.S. Kulp, M.G. Knize and S.L. McCutchen-Maloney, PhIP metabolites in human urine and breast cancer cells: Implications for the study of individual variation of carcinogen metabolism and chemoprevention through dietary interactions California Breast Cancer Research Program, March 8-10, 2002.

K.S. Kulp, M.G. Knize, S.L. McCutchen- Maloney, and J.S. Felton, "PhIP metabolites in human urine and human cancer cells: Implications for the study of individual variation of carcinogen metabolism and chemoprevention through dietary interactions" UC Davis Cancer Research Symposium; Sacramento, CA; October 2001

4/4/01 UC Berkeley Dept. of Epidemiology- Seminar (Risks of Overcooked Foods)

6/15/01 National Cancer Institute (Bethesda)- Seminar (Are Carcinogens in Food a risk for human Health?)

9/24/01 Environmental Mutagen Society Breast Cancer Conference- Symposium talk (Human Exposure to Heterocyclic Amine Food Mutagens/Carcinogens: Relevance to Breast Cancer)

11/12/01 8th International Conference on Carcinogenic/Mutagenic N-Substituted Aryl Compounds, Washington DC. (Factors affecting human heterocyclic amine intake and the metabolism of PhIP)

11/12/01 N-Substitute Aryl Compound International Meeting- Symposium talk (25 years of research on heterocyclic amines: What can we say about their impact on human cancer?)

3/2/02 Univ of Arkansas Colon Cancer Symposium- Symposium talk (Role of heterocyclic amines in colon and prostate cancer)

3/5/02 National Center for Toxicological Research- Seminar (Do Heterocyclic Amines pose a Human Risk)

Funding Applied for:

Determining the carcinogenic significance of heterocyclic amines , NIH Program Project Grant , funded.

Quantifying the impact of diet on carcinogen exposure , Exposure methods for cancer research, NIH, CA-01-018, funded.

Determining the effects of preventive foods on the absorption and metabolism of a mammary carcinogen in humans , Department of Defense, BCRP, not funded.

"Gel Microdrop Capture/Detection of Tumor Cells" submitted to the NCI RFA, "Development of High-Yield Technologies for Isolating Exfoliated Cells in Body Fluids", PAR-01-019, pending.

CONCLUSIONS:

During the second year of the grant we continued a study that will to determine the stability of PhIP metabolism within an individual over time and have investigated the effect of soy on PhIP metabolism in humans and have expanded our understanding of the effect of these compounds on prostate cells in culture. We have discovered that PhIP metabolism is affected by diet and lifestyle factors and may determine that soy affects PhIP metabolism. We have improved our urine analysis method and are working to identify PhIP metabolites in prostate cells.

We continue to have some problems with LC/MS/MS quantitation. This is not a trivial problem and has been reported to be an issue by many labs. We have changed some of the LC/MS and sample extraction procedures to improve sample variability

This research uses state-of-the-art instrument measurement methods to support conclusions about the role of diet and prostate cancer in humans. Although still preliminary, our results indicate that other components of the diet, such as broccoli and soy, may have an effect on the metabolism of a commonly-occurring food carcinogen. Our investigations of the metabolism of PhIP and its intermediates and their effect on cellular response in prostate cancer cells may explain why this carcinogen specifically causes tumors of the prostate. It is possible that there are unique metabolic pathways present in prostate cells that produce a reactive intermediate that specifically causes DNA damage in the prostate.

We are on target to continue the work proposed in this grant.

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APPENDICES:

M.G. Knize, K.S. Kulp, C.P. Salmon, G.A. Keating and J.S. Felton, Factors affecting the human heterocyclic amine intake and the metabolism of PhIP . *Mutation Research*, in press.

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FACTORS AFFECTING HUMAN HETEROCYCLIC AMINE INTAKE AND THE METABOLISM OF PHIP.

Mark G. Knize*, Kristen S. Kulp, Cynthia P. Salmon, Garrett A. Keating and James S. Felton.

Biology and Biotechnology Research Program, P.O Box 808, Lawrence Livermore National Laboratory, Livermore CA, 94551-9900.

* 925 422-8260 knize1@llnl.gov

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isothiocyanates shown to induce Phase I and Phase II metabolism *in vitro*, may affect both the rate of metabolite excretion and the metabolic products of a dietary carcinogen. This newly developed methodology will allow us to assess prevention strategies that reduce the possible risks associated with PhIP exposure.

1. Dietary Intake and Heterocyclic Amine Carcinogens

Human epidemiologic and animal studies have shown that diet has a role in the etiology of human cancer. Diet is one aspect of an individual's lifestyle that may be practically modified. Therefore it is important to quantify dietary exposures to understand an individual's risk for cancer and to identify habits or practices that increase or decrease an individual's risk. Although complex, the interactions between the myriad different components in the whole diet may be a critical factor in determining the likelihood of cancer initiation.

There is general consensus that potent genotoxic carcinogens are produced in meat during cooking at high temperatures. The demonstrated mutagenicity of these compounds in bacteria [1], cells in culture [2, 3] and mice [4, 5], support the many studies of carcinogenicity in mice and rats [1, 6]. Mechanistic data show DNA adducts in rodents and humans consuming these compounds at low doses [7].

Although, the role of heterocyclic amines in cancer initiation has been well-established in animals, much less in known about the effect of heterocyclic amine exposure on tumor development in humans. The presence of heterocyclic amines in commonly consumed commercially

Gender differences are known in human bladder cancer, with males being more sensitive [18]. For well-done meat and colorectal cancer, there was a non-significant two-fold increase in males, but not in females [19]. Are mixed gender studies of aromatic amine carcinogenesis confounded? Gender differences are just beginning to be investigated in laboratory studies and need further investigation.

Recently epidemiologists have begun investigating possible links between well-done meat consumption and cancer risk. Several epidemiology studies have reported an increased risk of cancer associated with subject groups that prefer well-done meat. In 1998, Zheng *et al.* described a significant dose-response relationship between doneness levels of meat and breast cancer risk; women who preferred well-done hamburger, steak and bacon had a 4.6 fold greater risk of breast cancer than did women who preferred meats cooked "rare" or "medium" [20]. Other studies reported an increased risk of colorectal adenomas with increased well-done meat consumption [21, 22]. Lung cancer risk has also been related to the consumption of fried, well-done meat [23]. Other studies, however, have shown either equivocal associations with well-done meat and cancers of the prostate gland [24] or negative associations with cancers of the breast [25, 26], colon or rectum [11].

In all of these studies, heterocyclic amine exposure levels are based upon answers to dietary questionnaires. However, the formation of heterocyclic amines in foods depends on many cooking variables and

interpretation of meat doneness are responsible for a great deal of variation in heterocyclic amine amounts, especially for PhIP in chicken.

For example, marination of meat is a cooking method generally not accounted for in dietary questionnaires for heterocyclic amine exposure assessment. Figure 1 shows the formation of PhIP in chicken breast meat as a function of weight loss during cooking. Analysis was performed on meats grilled, fried, or broiled in our laboratory or on meat samples that had been sent to us previously cooked [30]. Only when chicken breast is cooked to extreme dryness (weight losses of 40% or more), do PhIP levels increase to the very high levels occasionally found. Because weight loss and the perceived dryness of the food is used as a measure of cooking doneness, it is apparent from Figure 1 that determining when samples are "done" can have a great effect on PhIP levels. Also shown in Figure 1 is the effect of marinating on PhIP formation. As we have described previously, marinating before grilling greatly reduces PhIP levels in chicken [31]. Notably, in samples cooked to the same degree of weight loss, PhIP levels are up to 10-fold less in the marinated samples. These results emphasize the extreme differences in PhIP levels that can occur as a result of different cooking methods.

Another uncertainty surrounds the heterocyclic amine databases used to construct exposure categories. Most epidemiologic studies of heterocyclic amines use relationships between heterocyclic amine concentrations and doneness level derived from laboratory cooking studies. However, heterocyclic amine levels in meats obtained from homes have varied considerably from the laboratory data. In a study of

amounts of PhIP formed in the two meat types are not significantly different (p=0.36) from each other.

The highly variable concentrations observed in these home-cooked samples, especially for PhIP in very well-done chicken, may contribute to the contradiction of white-meat associated low cancer rates and high heterocyclic amine exposure. Using high heterocyclic amine values reported in an early study of laboratory-cooked chicken *[30]*, Byrne et al. concluded that chicken prepared by grilling, broiling, or pan-frying are the three foods that most reliably predict PhIP exposure [27]. However, based upon the results presented in Figure 2, as well as analysis of meat cooked in restaurants [32], we believe that the levels of PhIP are similar in chicken and beef when the meat is cooked in typical households. In the same study by Byrne et al., broiled fish was identified as the fourth "predictor of PhIP exposure". In studies of fish cooked to the doneness usually eaten in the US or Sweden, there is little evidence in support of the conclusion that broiled fish contains more PhIP than beef steaks [33, 34]. The research group that reported large amounts of PhIP in well-cooked salmon *[35]* found no PhIP in another grilled fish type in a follow-up study that compared laboratory grilled beef, pork (bacon), and fish [36]. Yet the latter study is not often considered when assessing dietary intake.

Based on these observations it is apparent that quantifying human heterocyclic amine exposure is not a simple task. Formation of heterocyclic amines in meat during cooking is highly dependent upon cooking method and doneness levels. Individual exposure depends upon

to monitor changes in metabolic enzyme activity. We developed a method for quantifying PhIP metabolites in human urine following a single meal of well-done meat.

Pioneering work in *in vivo* human metabolism examined the relationship of urinary excretion of the unmetabolized parent compound and the dose received in well-done hamburgers [38, 39]. Other studies demonstrated the presence of PhIP and PhIP conjugates in human urine, but in these studies the urine was first treated with acid to hydrolyze the Phase II metabolic conjugates to the parent amine. These investigations showed that PhIP is bioavailable in humans, but did not give information about specific metabolic pathways [40-42]. Most recently, specific results about the identity of human PhIP metabolites were obtained in studies that investigated human PhIP metabolism following administration of [^{14}C]-labelled PhIP to patients undergoing cancer surgery [43-45]. Surprisingly, the relative amounts of human urinary metabolites were unlike those of rodents and more like those of dogs [44]. These studies identified four major human PhIP metabolites: *N*^{2}-OH-PhIP-*N*^{2}-glucuronide, PhIP-*N*^{2}-glucuronide, PhIP-4'-sulfate, and *N*^{2}-OH-PhIP-*N*3-glucuronide. Based on the metabolite identification, we developed a solid-phase extraction, LC/MS/MS method that quantifies the four known PhIP metabolites in human urine, following a single meal of well-cooked chicken [46]. Chicken is used in this assay because we can produce PhIP in overcooked chicken without a concomitant amount of other known heterocyclic amines. Because the PhIP is formed naturally in the chicken at levels that represent possible dietary exposures, we can apply this method to characterize PhIP metabolism in normal, healthy volunteers.

non-meat foods and beverages with the cooked chicken. Control urine was collected before eating the chicken and samples were collected for 24 hours after in increments of 6 hours.

Urine samples were prepared according to Kulp et al. [46]. Briefly, an internal standard of deuterium labeled N-OH-PhIP-N^2-glucuronide was added to five ml samples of urine. The urine was then applied to a pre-conditioned macroporous polymeric column. Metabolites were eluted with methanol and the methanol fraction evaporated to dryness under nitrogen. The metabolites were re-dissolved in 0.01M HCl and high molecular weight contaminants were removed by filtering the solution through a centrifugal filter at 3000 × g overnight. The filtrate was applied to a pre-conditioned benzenesulfonic acid column and the column washed with a mixture of methanol and 0.01M HCl. The metabolites were eluted onto a coupled C18 column with 0.05 M ammonium acetate, pH 8. The C18 column was washed with 5% (v/v) methanol/H_2O and eluted from the C18 column with 50% (v/v) methanol/H_2O. The metabolites were dried under nitrogen and 1 ml urine equivalents were injected into the LC/MS/MS in a volume of 20 µl.

Metabolites were detected with an ion trap mass spectrometer (model LCQ, Finnigan, San Jose, CA) in the MS/MS positive ion mode using an electrospray interface as published [47]. Alternating scans were used to isolate $[M+H]^+$ ions at mass 417, 401, and 321 for natural PhIP metabolites, and 422, for the pentadeutero-labeled internal standard metabolite. Collision energy was 25%. Daughter ions were detected at appropriate masses: 241 (M+H-glucuronic acid) and 225 (M+H-glucuronic

improved in several ways. Heavy-isotope labeled metabolites are necessary for recovery determination of the N-hydroxy-N3 PhIP glucuronide, PhIP N2 glucuronide, and PhIP-4'-sulfate. Additional PhIP metabolites are known to be present in human urine but have not yet been fully characterized. Although the unknown metabolites occur in smaller amounts than the four detected, quantifying these metabolites would provide a more complete picture of biological fate of the PhIP ingested in the chicken meal. Recently available mass spectrometers have about 10-fold more sensitivity than the current model, which might lead to improved peak signal thereby reducing injection-to-injection variability.

A biomarker of heterocyclic amine exposure is still needed

To understand the effect of heterocyclic amine exposure on human health, we need to be able to assess actual exposures from meat prepared as it is commonly eaten in homes. Although measuring urine metabolites is one way of characterizing metabolism patterns, the metabolites excreted in the urine only represent exposures that may have occurred in the previous 24h. The optimal biomarker of exposure would integrate heterocyclic amine exposures over time. Hair has been investigated as a marker of PhIP exposure over the previous 6 months [48].

Aflatoxin exposure assessment is similar to the heterocyclic amine exposure problem in meat. It sometimes occurs in only some foods, so the food contamination and amount eaten are both important for dose

not available fully characterize the relationship between heterocyclic amines and human cancer.

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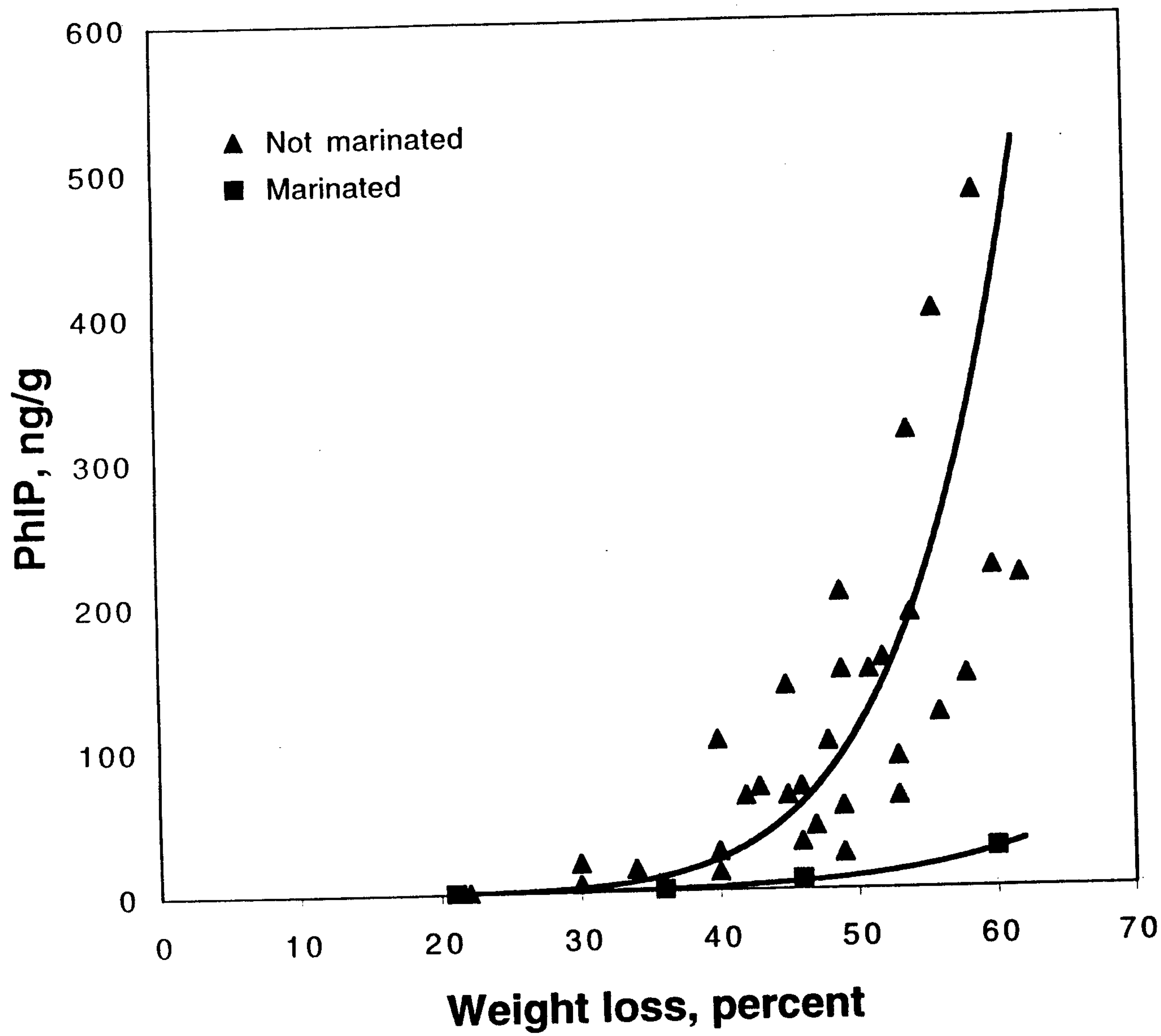
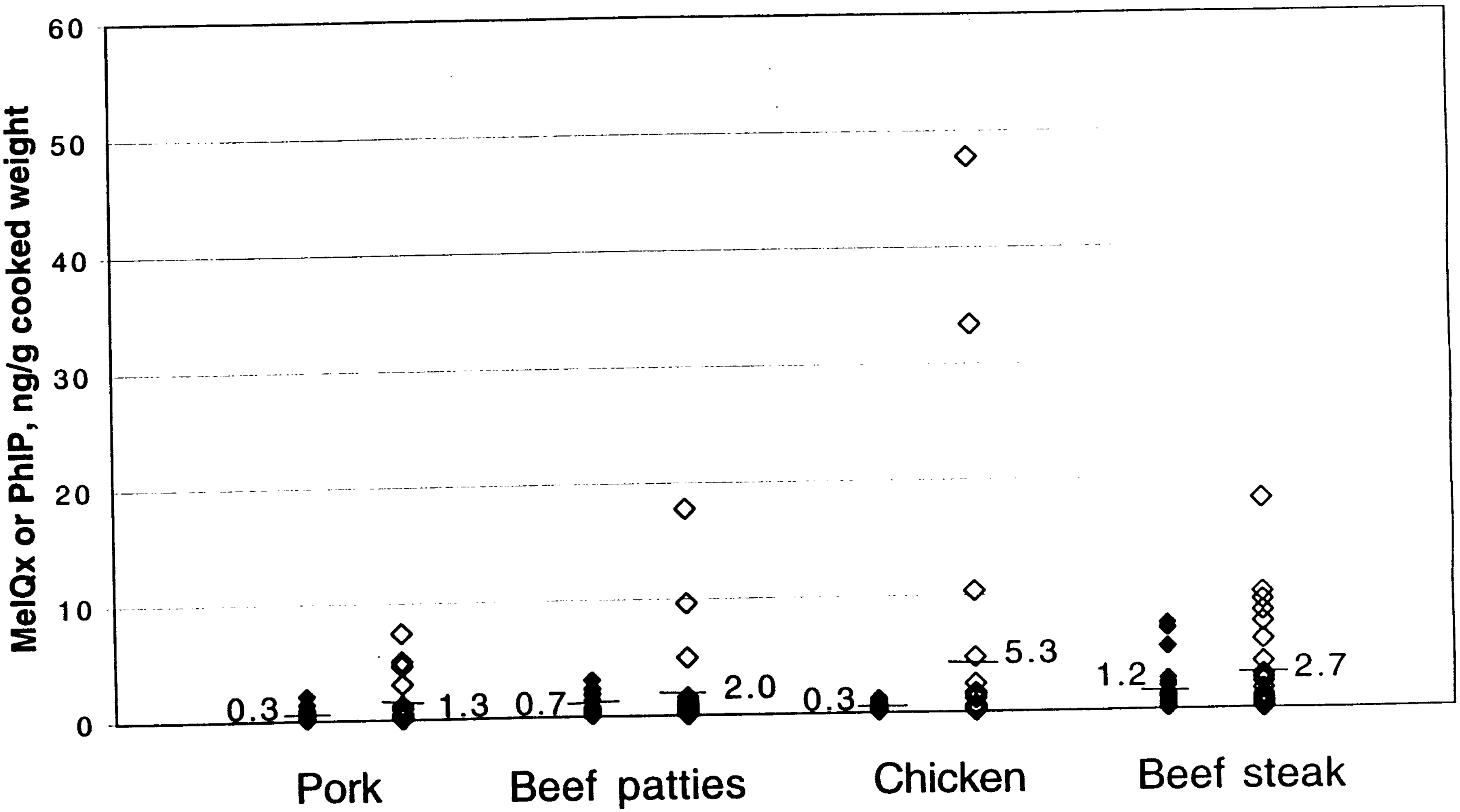
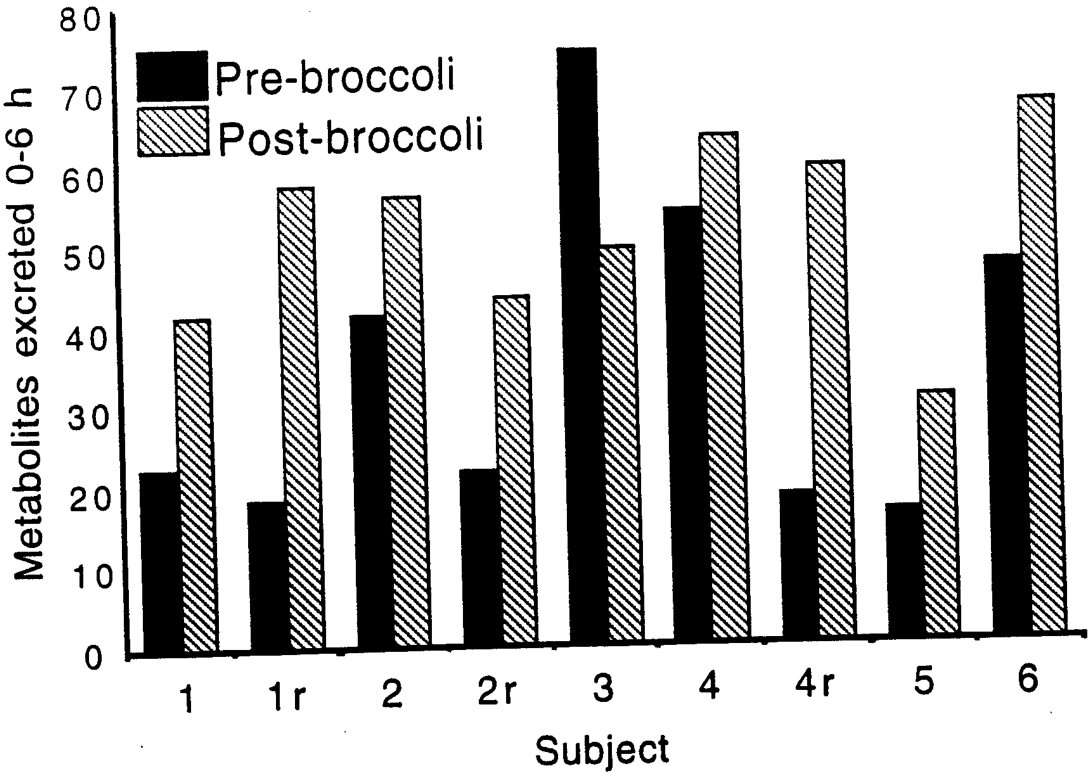
Meat	PhIP	MeIQx	All heterocyclic amines
Chicken	25% (5/20)	20% (4/20)	15% (3/20)
Beef steak	22% (7/32)	15% (5/32)	12.5% (4/32)
Pork	50% (10/20)	35% (7/20)	30% (6/20)
Beef patty	30% (6/20)	25% (5/20)	25% (5/20)



Not marinated
Marinated
PhIP, ng/g
600
500
400
300
200
100
0
0
10
20
30
40
50
60
70
Weight loss, percent


MeIQx or PhIP, ng/g cooked weight
60
50
40
30
20
10
0
0.3
1.3
0.7
2.0
0.3
5.3
1.2
2.7
Pork
Beef patties
Chicken
Beef steak



80
70
60
50
40
30
20
10
0
Metabolites excreted 0-6 h
Pre-broccoli
Post-broccoli
1
1r
2
2r
3
4
4r
5
6
Subject

Environmental and Molecular Mutagenesis 39:112–118 (2002)

Human Exposure to Heterocyclic Amine Food Mutagens/Carcinogens: Relevance to Breast Cancer

James S. Felton,* Mark G. Knize, Cynthia P. Salmon, Michael A. Malfatti, and Kristen S. Kulp

Molecular and Structural Biology Division, Lawrence Livermore National Laboratory, Livermore, California

Heterocyclic amines produced from overcooked foods are extremely mutagenic in numerous in vitro and in vivo test systems. One of these mutagens, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), induces breast tumors in rats and has been implicated in dietary epidemiology studies as raising the risk of breast cancer in humans. Efforts in our laboratory and others have centered on defining the exposure to PhIP and other dietary mutagens derived from cooked food. We accomplish this by analyzing the foods with a series of solid-phase extractions and HPLC. We have developed an LC/MS/MS method to analyze the four major human PhIP metabolites (sulfates and glucuronides) following a single meal containing 27 μg of cooking-produced PhIP in 200 g of grilled meat. Although the intake of PhIP was similar for each of eight women, the total amount excreted in the urine and the metabolite profiles differed among the subjects. It appears that adsorption (digestion) from the meat matrix, other foods in the diet, and genetic differences in metabolism may contribute to the variation. The four major metabolites that can be routinely assayed in the urine are N^2-OH-PhIP-N^2-glucuronide, PhIP-N^2-glucuronide, 4′-PhIP-glucuronide, and N^2-OH-PhIP-N3-glucuronide. This work is suited to investigate individual exposure and risk, especially for breast cancer, from these potent dietary mutagens. Environ. Mol. Mutagen. 39:112–118, 2002. Published 2002 Wiley-Liss, Inc.†

Key words: dietary mutagen; heterocyclic aromatic amines; glucuronide; PhIP; tumorigenicity; chemoprevention

INTRODUCTION

The cooking, heat processing, and pyrolysis of protein-rich foods result in the formation of a group of structurally related heterocyclic aromatic amines that are potent mutagens in a number of assay systems. These same compounds produce tumors at multiple organ sites (including sites of important neoplasms in North Americans) in both male and female mice and rats [Shirai et al., 1997; Sugimura, 1997]. Furthermore, 100% of nonhuman primates given one of these heterocyclic amines (2-amino-3-methylimidazo[4,5-*f*]quinoline [IQ]) developed hepatocarcinomas after a very short latency period [Adamson et al., 1990, 1994]. Epidemiology data from a number of studies in the United States, New Zealand, South America, and Europe suggest a good correlation of meat consumption with cancer risk in humans. At a recent American Association for Cancer Research (AACR) meeting, there were four positive reports (three for breast cancer) relating high meat intake and genetic susceptibility with human cancer (8.2 relative risk for breast cancer when low Sult1A1 [Zheng et al., 2000], 3.5 odds ratio for breast cancer when rapid NAT2 [Visvanathan et al., 2000], and 1.9 odds ratio for breast cancer in the highest exposure group [Sinha et al., 2000]). It is now clear from a number of recent studies that these heterocyclic amines are present in the diet at higher levels than were originally anticipated [Knize et al., 1998]. The usual factor correlated with meat consumption and cancer occurrence is fat intake, but clearly, heterocyclic amine intake also correlates well and has a plausible genotoxic mechanism, leading directly to DNA binding, mutation, and cancer initiation.

Abbreviations: AαC, 2-amino-9*H*-pyrido[2,3-*b*]indole (CAS no. 26148-68-5); 4,8-DiMeIQx, 2-amino-3,4,8-trimethylimidazo[4,5-*f*]quinoxaline (CAS no. 95896-78-9); 8-MeIQx, 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (CAS no. 77500-04-0); DMIP, 2-amino-1,6-dimethylimidazo[4,5-*b*]pyridine; IFP, 2-amino-1,6-dimethylfuro[3,2-*e*]imidazo[4,5-*b*]pyridine; IQ, 2-amino-3-methylimidazo[4,5-*f*]quinoline (CAS no. 76180-96-6); IQx, 2-amino-3-methylimidazo[4,5-*f*]quinoxaline (CAS no. 108354-47-8); MeIQ, 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline (CAS no. 77094-11-2); PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (CAS no. 105650-23-5); TMIP, 2-amino-1,5,6-trimethylimidazo[4,5-*b*]pyridine; CHO, Chinese hamster ovary cells.

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*Correspondence to: James S. Felton, Molecular and Structural Biology Division, L-452, Lawrence Livermore National Laboratory, Livermore, CA 94551. E-mail: felton1@llnl.gov

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Given these very compelling data, it is important to determine the extent to which these dietary mutagens/carcinogens contribute to human breast cancer incidence and to devise strategies to limit their impact. In this report we discuss the exposure with emphasis on heterocyclic amines in restaurant-cooked foods. We also discuss the risk of exposure to heterocyclic amines, metabolism with emphasis on glucuronyl transferases, urine metabolite biomarkers, and their possible use in evaluating risk for breast cancer from these carcinogens found in cooked meat.

HETEROCYCLIC AMINE ANALYSIS OF FOODS

Twenty years ago, the chemicals responsible for the observed mutagenic activity in cooked meat were unknown. Discoveries in the dietary heterocyclic amine field date back more than 24 years [Sugimura et al., 1977]. Dr. Sugimura and his group first showed that cooking of meat and fish produced potent bacterial mutagens [Sugimura et al., 1977]. Dr. Kasai, working with Drs. Sugimura and Nishimura, described the structure of the first mutagen isolated from cooked meat (IQ) [Kasai et al., 1981]. Shortly after this initial work, our group quantified the level of mutagenic activity in numerous food types in the Western diet. We later isolated and identified from cooked ground beef IQ, MeIQx, and, for the first time, DiMeIQx and PhIP [for review, see Felton et al., 1986; Felton and Knize, 1991; Felton, 1994]. We also determined that PhIP was present at approximately 10-fold higher mass amounts than that of the other heterocyclic amine mutagens [Felton et al., 1986]. Our scientists partnered with researchers from the Nestlé Ltd. Research Centre to develop analytical methods for the practical detection of heterocyclic amines in foods, to determine the foods and cooking conditions responsible for human exposures [Gross and Grüter, 1992; Knize et al., 1992].

A few years later, liver tumors were observed in cynomolgus monkeys fed IQ [Adamson et al., 1990]. With the discovery of mutagenic responses of these heterocyclic amines in multiple genotoxic assay systems, and carcinogenicity responses in both sexes and multiple organs of rats [Sugimura et al., 1988], mice [Ohgaki et al., 1987; Esumi et al., 1989], and primates [Adamson et al., 1990], it became clear that these compounds had a potentially important impact on human health and, particularly, on cancer risk [Sugimura, 1997]. In one of the early human epidemiological studies, Schiffman and Felton [1990] described an increased relative risk for colon cancer for individuals consuming fried meats.

HUMAN RISK TO HETEROCYCLIC AMINES

Data have been reported on the levels of the heterocyclic amines in the diet [Fennema and Hall, 1990; Layton et al., 1995]. Several early studies on the epidemiology of these compounds [Gerhardsson de Verdier et al., 1991; Steineck et al., 1993; Goldbohm et al., 1994] showed a relationship between meat consumption and human cancer (see above for more recent epidemiology studies related specifically to breast cancer). Human risk, based on linear extrapolation of TD_{50} calculations from mouse, rat, or primate tumor data, and on mean estimated mutagen exposures for the U.S. population, suggests potential risks of 10^{-5} to 10^{-3} [Gaylor and Kadlubar, 1991; Layton et al., 1995]. Although these risk calculations contain many generalizations and assumptions, nevertheless they indicate that human risk from dietary ingestion of these heterocyclic amines may be significant. These risk estimates need to be supported or refuted using much more rigorous data and linked to specific human subpopulations that may be more susceptible or "at risk" than is the average population.

MUTAGENS IN THE DIET

Analysis of *Salmonella* mutagens in major sources of cooked protein in the American diet (based on USDA and USDHEW surveys) showed significant mutagen content in beef, eggs, pork, ham, and bacon, and lesser amounts in chicken and fish (fried or broiled) [Bjeldanes et al., 1982a]. Tofu, beans, cheese, and some fish, when cooked under similar conditions, produced low or negligible mutagenic activity [Bjeldanes et al., 1982b]. Mutagen isolation was improved by aqueous extraction at pH 2 followed by absorption/elution of mutagens on XAD-2 resin [Bjeldanes et al., 1982a]. Chromatographic purification of mutagens from 100-kg batches of fried beef was combined with high-resolution mass spectrometry and NMR techniques to show the presence of at least 10 separable mutagens. The largest amount of mutagenicity was provided by MeIQx (~35% of total activity), which is present at about 1 μg/kg original fresh weight of beef. Additional major mutagens were 4,8-DiMeIQx (0.5 μg/kg) and PhIP (15 μg/kg). Several other mutagens were present, including IQ (0.02 μg/kg), MeIQ (at <0.01 μg/kg), and TMIP (0.5 μg/kg) [Felton et al., 1984]. More recently, analytical methods were further improved with the development of GC/MS techniques and solid-phase extraction with HPLC analysis [Gross and Grüter, 1992; Knize et al., 1992]. This work has led to the finding that heterocyclic amine content in foods is significantly higher than was originally anticipated.

Mutagen production in beef, chicken, and pork has been examined at different temperatures. Even though total mutagenic activity increases dramatically with increasing temperature, chromatographic analysis shows that the relative amounts of the mutagenic peaks are similar [Knize et al., 1985]. Mutagen profiles from chicken breast meat (ground and then fried) is similar to, but not identical with, the beef mutagen profile [Knize et al., 1988]. Our early analysis (a collaboration with the group at Wageningen University, The Netherlands) of a complete human diet, with foods and amounts taken from a dietary survey and cooked under

TABLE I. Heterocyclic Amines in Restaurant Foods[a]

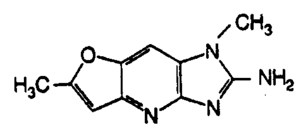
Sample	Restaurant—doneness	IFP	MeIQx	PhIP	DMIP	TMIP	DiMeIQx
Top sirloin	A—well-done	nd	1.2[b]	1.8	nd	nd	nd
New York steak	A—well-done	nd	0.12	0.86	nd	nd	nd
Pork chop	A—unspecified	nd	0.4	2.4	nd	nd	nd
Beef (French-dip sandwich)	A—unspecified	nd	nd	nd	nd	nd	nd
New York steak	B—well-done	7.0	1.3	7.7	7.2	1.5	0.77
Tenderloin steak #1	C—well-done	7.6	1.9	16	nd	nd	nd
Tenderloin steak #2	C—well-done	21	0.67	49	nd	nd	nd
Top sirloin steak	D—well-done	3.3	2.0	7.8	nd	nd	nd
London broil steak	C—well-done	46	3.0	182	3.4	nd	nd
Prime rib	C—well-done	nd	nd	nd	nd	nd	nd
Beef (fajitas)	D—unspecified	1.4	0.93	1.7	0.59	nd	0.06
Au jus gravy	A—unspecified	nd	nd	nd	nd	nd	nd

[a]ng heterocyclic amine/g cooked meat; nd, not detected.
[b]Average of duplicate analyses of a single sample.

"household" conditions, also shows chromatographic types and amounts of mutagens similar to those of fried beef [Alink et al., 1988]. More recent studies show that the amounts of these compounds formed increase exponentially with temperature, and the ultimate levels attained are dependent on cooking method, cooking time, cooking temperature, and protein source [Knize et al., 1994]. In fact, the levels in some foods, such as chicken, can reach hundreds of parts per billion [Sinha et al., 1995]. In general, these compounds are formed at surface temperatures in excess of 150°C and are found in all well-done broiled, grilled, or fried muscle meat products, including fish, beef, pork, and chicken. These heterocyclic amines have also been reported in cigarette smoke [Manabe et al., 1991] and wine and beer [Manabe et al., 1993], although these findings have not yet been confirmed in other laboratories. These results clearly indicate that cooked meats are the major source of heterocyclic amines in the human diet.

Most recently, we have analyzed restaurant-cooked foods to see how the levels of heterocyclic amines compare to those found from laboratory and home cooking. In Table I, we show a large range in heterocyclic amine content from different meats. In one restaurant, the level of PhIP was almost 200 ppb in London broil beef ordered well-done. In most cases, the levels were at least 10-fold below this highest level. Chicken, especially that grilled from a Mexican restaurant, was significantly high for a number of the heterocyclic amines. This study shows that exact concentrations of the heterocyclic amines in these cooked foods will be difficult to determine based only on questionnaires of doneness preference. However, it is clear from this study that significant amounts of heterocyclic amines can be consumed from eating commercially cooked well-done meats.

The identification of new mutagens from cooked meats has been difficult but successful. Several new mutagens have been identified, with structures consisting of two fused rings and either two or three methyl groups (DMIP and TMIP). Recently, a new mutagen with an imidazo-furo-pyridine structure has been found in a variety of meats (Table I) and its structure



IFP
(2-amino-1,6-dimethylfuro[3,2-*e*]imidazo[4,5-*b*]pyridine)

Fig. 1. Structure of 2-amino-1,6-dimethylfuro[3,2-*e*]imidazo[4,5-*b*]pyridine (IFP).

has been characterized as 2-amino-(1,6-dimethylfuro[3,2-*e*])imidazo[4,5-*b*]pyridine (see Fig. 1 for the structure). Its role in breast cancer and other human tumor sites is unknown at this time, but its potent mutagenic activity and its structural similarity to PhIP make investigation of its biological effects a priority.

HETEROCYCLIC AMINE MUTAGEN METABOLISM

The metabolism of PhIP and 4,8-DiMeIQx, two of the most mass-abundant heterocyclic amines, differing greatly in their mutagenic response in cultured CHO cell and Salmonella mutagenic responses, were investigated in both in vivo and in vitro rodent experiments. PhIP is metabolized to two major metabolites by mouse liver microsomes, one of which is a direct-acting mutagen (N-OH-PhIP) to *Salmonella* and CHO cells. The other metabolite is hydroxylated at the 4′ position of the phenyl ring and appears to be a detoxification product [Turteltaub et al., 1988]. Thus, it is important to understand factors that favor formation of one or the other of these metabolites because the ratio will affect the level of reactive intermediates available for DNA binding (adduct formation) and mutation.

In Aroclor 1254–induced C57BL/6 mice, PhIP is excreted almost completely in 24 hr, with some differences in its uptake kinetics between oral and intraperitoneal administration. The urine shows at least six metabolites, with less than 10% of the dose excreted as the unaltered parent compound [Turteltaub et

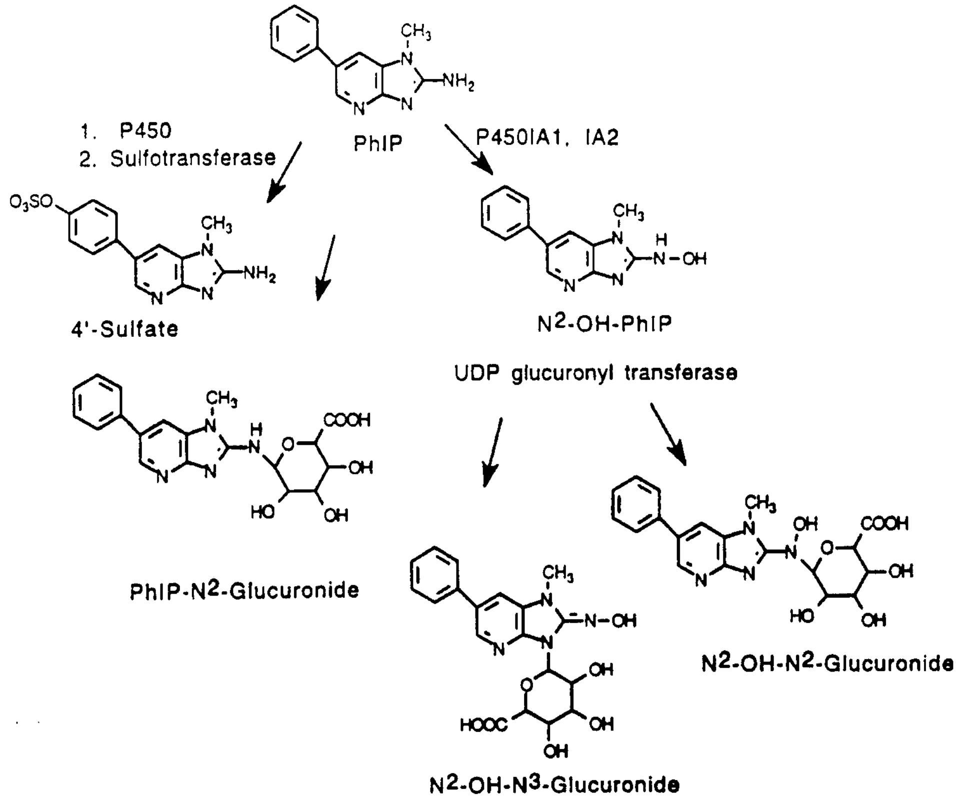


Fig. 2. Structures of PhIP metabolites and pathways of their formation. These metabolites are identified in human urine after consumption of a single meal of well-done chicken. The major metabolites are N^2-OH-PhIP-N^2-glucuronide (conjugation of an active metabolite) and PhIP-N^2-glucuronide (a conjugated detoxification product).

al., 1989]. 4,8-DiMeIQx is metabolized to eight metabolites by microsomes in vitro. Two of these have been identified as nitro-4,8-DiMeIQx, which is possibly a degradation product of N-hydroxy-4,8-DiMeIQx and 8-hydroxymethyl-4,8-DiMeIQx [Turteltaub et al., 1988].

In rats, seven major 4,8-DiMeIQx metabolites were detected in the urine and feces. Germ-free rats, having no intestinal microflora, produced the same group of metabolites in both the urine and feces as did the normal rats [Knize et al., 1989]. This suggests that microbial metabolism is not a significant factor in the metabolism of this mutagen.

It seems clear from in vitro studies that acetylation is required for the formation of active electrophiles of IQ and MeIQx, but not of PhIP. Mutagenicity of N-hydroxy-PhIP depends somewhat on bacterial sulfotransferase activity but not on acetylation. IQ and MeIQx, but not PhIP, were significantly less mutagenic in *Salmonella* strains that had a deficiency in *N*-acetyltransferase activity [Holme et al., 1989; Buonarati et al., 1990]. In collaboration with Dr. Josephy (Guelph University, Canada), we also showed that strains overexpressing *N*-acetyltransferase were more responsive to IQ but not to PhIP (unpublished data). It appears that N-OH intermediates of these amines have different requirements for conjugation and these differences may explain variable responses in CHO cells and tissue-specific carcinogenicity differences. Further, human tissue cytosols catalyze both N:O-acetylation and N:O-sulfation, but the in vivo rates of metabolism have yet to be determined. More recently, strains overexpressing sulfotransferase showed the biggest increase in mutation and cytotoxicity with PhIP [Wu et al., 2000], but nowhere near the large response seen with IQ when acetyltransferase is overexpressed [Wu et al., 1997]. These differences need to be explored to understand individual differences in biological response, especially in the tumor targets for these heterocyclic amines, such as breast tissue.

METABOLISM IN HUMANS

Following cytochrome P4501A2 activation of the parent amine to the corresponding 2-hydroxyamino intermediate, a number of conjugating reactions can take place [Boobis et al., 1994; Edwards et al., 1994]. For PhIP, the N-hydroxy intermediate can be esterified by sulfotransferase and/or

acetyltransferase to generate the highly electrophilic O-sulfonyl and O-acetyl esters [Buonarati et al., 1990; Ozawa et al., 1994]. Most interestingly, human metabolism of PhIP is dominated by glucuronidation (see Fig. 2) [Malfatti et al., 1999]. In addition, understanding of glucuronidation by a family of enzymes called the UDP-glucuronosyltransferases (UGTs) is needed. These enzymes exist as a number of different isoforms [King et al., 1996; Mackenzie et al., 1997; Strassburg et al., 1998], but the UGT1A subfamily contributes to the biotransformation of amines and PhIP, respectively [Green and Tephly, 1998; Nowell et al., 1999]. Microsomes containing the UGT1A1 isozyme have the highest capacity to convert *N*-hydroxy-PhIP to *N*-hydroxy-PhIP-N^2-glucuronide, the most abundant metabolite in human urine formed from PhIP [Malfatti et al., 2001]. In contrast, UGT1A9 produced *N*-hydroxy-PhIP-*N*3-glucuronide at the highest rate. Thus, the distribution and prevalence of these isozymes in the body may determine the rate and type of detoxification of PhIP and, ultimately, the target tissue for mutations and cancer.

Both the N^2 and the *N*3 positions on PhIP are glucuronidated directly (most likely these are nonreactive intermediates) or the glucuronidation occurs on the N-hydroxy intermediates. This can be envisioned as a direct detoxification pathway (see Fig. 2) [Styczynski et al., 1993; Kaderlik et al., 1994]. These glucuronides and the 4′ sulfation product on the phenyl ring of PhIP can be accurately measured in human urine using LC/MS/MS, after a single meal of cooked well-done meat [Kulp et al., 2000]. The ratios of these metabolites can be measured to understand individual differences in metabolism, and also can be used to determine whether chemopreventative agents can alter the metabolism of these mutagens (see below).

CHEMOPREVENTION IN HUMANS

With the ability to measure PhIP metabolites in humans, we can do intervention studies to determine whether chemopreventative agents, such as isothiocyanates in broccoli, can alter the metabolism (and possibly the risks) from exposure to these agents. Six volunteers were fed a single meal of well-done chicken after abstaining from broccoli or related cruciferous vegetables for 3 days. Metabolites were determined in urine collected in 6-hr increments. N^2-OH-PHIP-N^2-glucuronide was the primary PhIP conjugate detected in human urine after the chicken meal. The N^2-hydroxy-PhIP-*N*3-glucuronide, the PhIP-N^2-glucuronide, and the PhIP-4′-sulfate were the other major metabolites. After eating cooked broccoli for 3 days, the experiment with well-done chicken was repeated. As shown in Figure 3, metabolism of PhIP to the conjugates detected in the first 6 hr was increased statistically in all but one individual after the broccoli consumption. This suggests that components in the broccoli increased the rate of PhIP metabolism.

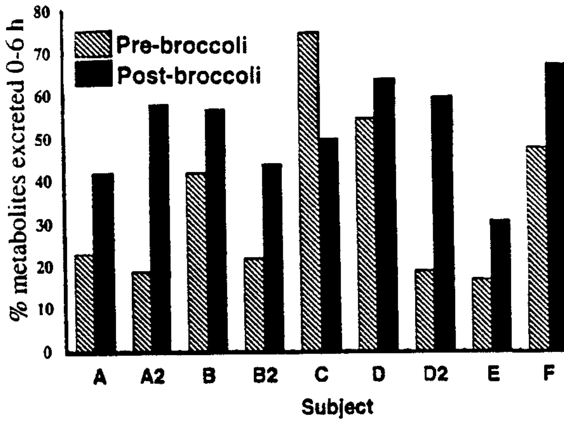


Fig. 3. Percentage of the measurable PhIP metabolites excreted in the 0–6 hr time period in six individuals after abstaining from broccoli-related cruciferous vegetables (pre-broccoli) or after consuming broccoli daily for 3 days (post-broccoli). Volunteers were given a single meal containing well-done chicken breast, cut into 2.5-cm pieces fried 25–35 min at an average pan temperature of 186°C. Total PhIP doses were 10–20 μg. Three individuals were assayed twice (2 represents repeat on the same individual 3 months later). The increase in excreted metabolites may be attributable to induction of phase II metabolizing enzymes by compounds such as isothiocyanates in the broccoli.

CONCLUSIONS

The investigation of the heterocyclic amines and their human intake is important for breast cancer research, for several reasons. From epidemiology studies, breast cancer is relatively high among women eating a Western diet, which is consistent with consumption of cooked meat (beef, chicken, pork, fish, and lamb) foods. One known heterocyclic amine, PhIP, consistently causes mammary tumors in rats, although IQ and Trp-P-2 induce mammary tumors as well in Sprague–Dawley and F344 rats, respectively. International studies show that PhIP is present in well-done meats, whether consumed in homes or in restaurants.

Under continuing investigation are the differences in heterocyclic amine metabolism, comparing and extrapolating rat tumorigenicity to humans. Extrapolation from high-dose animal experiments to the low doses found in human studies is difficult, but still there are compelling data to suggest heterocyclic amines may be good model compounds for investigation of breast cancer initiation in humans. Because we can measure carcinogenic metabolites in people, we can go forward with chemoprevention studies in humans, especially those relevant for breast cancer.

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Liquid chromatography–tandem mass spectrometry method of urine analysis for determining human variation in carcinogen metabolism

M.G. Knize*, K.S. Kulp, M.A. Malfatti, C.P. Salmon, J.S. Felton

Biology and Biotechnology Research Program, Lawrence Livermore National Laboratory, Livermore, CA 94550, USA

Abstract

We developed a solid-phase extraction LC–MS–MS method for the analysis of the four major metabolites of PhIP (2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine) in human urine after a meal of well-done chicken. Ten volunteers each ate either 150 or 200 g of well-done chicken breast containing 9–21 μg of PhIP. Among the individual volunteers there is 8-fold variation in the total amount of metabolites and 20-fold variation in the relative amounts of individual metabolites, showing individual differences in carcinogen metabolism. PhIP metabolites were also detected in urine from a subject consuming chicken in a restaurant meal, demonstrating the method's sensitivity after real-life exposures. Published by Elsevier Science B.V.

Keywords: Amines, heterocyclic aromatic; Aminomethylphenylimidazo[4,5-*b*]pyridine; Pyridines; Glucuronides

1. Introduction

PhIP (2-amino-1-methyl-6-phenylimidazo[4,5-*b*]-pyridine) is a potent mutagen and rodent carcinogen formed in meats from natural precursors during the cooking process. PhIP is found at the highest levels in grilled or fried meats and is frequently the most mass abundant heterocyclic amine produced during the cooking of beef, pork, and chicken [1–5], and in meats cooked by professional chefs and purchased in restaurants [6,7]. The human intake of PhIP varies with food type and cooking conditions and is estimated to range from nanograms to tens of micrograms per day, depending on individual dietary and cooking preferences [8]. Because humans are routinely exposed to varying amounts of these food-derived compounds there is a concern that they may play a role in human carcinogenesis.

PhIP must first be metabolized via Phase I and Phase II enzymes to exert its mutagenic and carcinogenic effect. During Phase I metabolism PhIP is oxidized to a hydroxylated intermediate, 2-hydroxyamino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (*N*-hydroxy-PhIP). *N*-hydroxy-PhIP is then converted to a more biologically reactive form via Phase II metabolizing enzymes, primarily the acetyltransferases or sulfotransferases. PhIP can also be hydroxylated at the 4′ position, forming 2-amino-1-methyl-6-(4′-hydroxy) phenylimidazo[4,5-*b*]pyridine (4′-hydroxy-PhIP). This hydroxylation does not produce an active intermediate. 4′-Hydroxy-PhIP can be conjugated by sulfation and glucuronidation to polar compounds that are readily excreted. Detoxification primarily involves glucuronidation. *N*-Hydroxy-PhIP can form stable glucuronide conjugates at either the N^2 or $N3$ positions. In addition, the parent compound can be directly glucuronidated at the N^2 and $N3$

*Corresponding author. Tel.: +1-925-422-8260; fax: +1-925-422-3915.

E-mail address: knize1@llnl.gov (M.G. Knize).

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positions. These glucuronides are not reactive and are excreted in the urine.

There is conclusive evidence that PhIP, a genotoxic carcinogen, is involved in tumorigenesis in animals. In rats and mice, dose-dependent tumor formation has been consistently demonstrated after PhIP administration, and the most common tumor sites in the rat appear to be colon, prostate, and breast [9–14].

Less is known about the role of PhIP in human carcinogenesis. Until recently, studies of human PhIP metabolism have been limited to hepatic microsomes or cells in culture. Pioneering studies in in vivo human metabolism demonstrated the presence of PhIP and PhIP conjugates in human urine, but in these studies the urine was first treated with acid to hydrolyze the Phase II conjugates to the parent amine. These investigations proved that PhIP is bioavailable in humans, but did not give information about specific metabolic pathways [15,16]. Specific results about the identity of human PhIP metabolites were obtained in studies that investigated human PhIP metabolism following administration of ^{14}C-labeled PhIP to patients undergoing cancer surgery. We recently described human PhIP metabolism in cancer patients receiving a single dose of radio-labeled PhIP in a capsule. These studies identified four major human PhIP metabolites: N^2-OH-PhIP-N^2-glucuronide, PhIP-N^2-glucuronide, PhIP-4′-sulfate, and N^2-OH-PhIP-N3-glucuronide [17].

In the present study we describe our development of a solid-phase extraction LC–MS–MS method for quantifying the four most abundant PhIP metabolites in human urine, following a meal of well-cooked chicken. We applied this method to characterize PhIP metabolism in 10 healthy individuals receiving a known dose of naturally produced PhIP. We have also extended this method to monitor metabolite excretion in a subject consuming chicken as part of a restaurant meal, demonstrating that our method is sensitive enough to detect PhIP metabolites after common real-life exposures.

2. Material and methods

2.1. Synthesis of N^2-OH-[$^2H_5$-phenyl]PhIP-N^2-glucuronide internal standard

The biological synthesis of deuterium labeled N-OH-PhIP-N^2-glucuronide was carried out in two steps as described previously [18]. Briefly, pentadeutero PhIP was reacted with baculovirus infected insect cell microsomes expressing human cytochrome P4501A2 (Gentest, Woburn, MA, USA) to produce the N-OH-[$^2H_5$-phenyl]PhIP intermediate. The reaction products were concentrated under N_2 and then isolated by HPLC using a Waters Alliance HPLC system equipped with a 5 μm, 220×4.6 mm TSK-Gel ODS-80 TM column (TosoHaas, Montgomeryville, PA, USA). Metabolites were detected using a Waters 990 photodiode array detector. The N-OH-[$^2H_5$-phenyl]PhIP was eluted at 1.0 ml/min using a gradient starting at 30% aqueous methanol, 0.1% triethylamine, pH 6, to 55% aqueous methanol, 0.1% triethylamine, pH 6, at 8 min. The methanol concentration was maintained at 55% from 8 to 20 min. After evaporation of the mobile phase, the yield of N-OH-[$^2H_5$-phenyl]PhIP from [$^2H_5$-phenyl]PhIP was approximately 40%.

Purified N-OH-[$^2H_5$-phenyl]PhIP was reacted with microsomes derived from the AHH-1 TK+/-human lymphoblastoid cell line which expresses human UDP-glucuronosyltransferase 1A1 (Gentest). The N-OH-[$^2H_5$-phenyl]PhIP-N^2-glucuronide was isolated and purified by HPLC using the conditions described above to give a 15% yield from N-OH-[$^2H_5$-phenyl]PhIP.

2.2. Study design

The study protocol was reviewed and approved by the Institutional Review Board for Human Research at Lawrence Livermore National Laboratory. Informed consent was obtained from each subject prior to beginning the study. The individuals participating were recruited from the local workforce, were males and females aged 22–45 years, in good health, non-smokers, and of normal weight.

2.3. Meat preparation and controlled dietary period

Boneless, skinless chicken breasts were cut into approximately 2.5 cm pieces and fried for 25 to 35 min in a non-stick coated pan sprayed with a vegetable-based cooking spray. Pan temperature averaged 186°C for the cooking period. At the end of the cooking time the chicken was white with some

browning. PhIP analysis was performed according to previously published methods [19].

Subjects were asked to abstain from meat consumption for 24 h prior to eating the well-done chicken breast. There were no other dietary restrictions. The first two study subjects were provided with 200 g chicken containing 105 ng/g PhIP. The total PhIP dose was 21 μg. Subjects three to eight were given 200 g of chicken containing 94 ng/g PhIP, for a total dose of 18.8 μg. The remaining two subjects were given 150 g of chicken containing 62 ng/g PhIP, for a total dose of 9.2 μg. All subjects were provided with other non-meat foods and beverages with the cooked chicken.

Control urine was collected before eating the chicken and for 24 h after in 6 h increments. Samples were refrigerated until analysis. Repeated analysis of these samples over prolonged periods of time (greater than 1 year) have shown no noticeable change in metabolite levels.

2.4. PhIP metabolite analysis after a restaurant meal

To test the sensitivity of detection of this method, one subject ordered and consumed chicken that was prepared as "chicken mango" at a local restaurant. The subject ate approximately 80 g of grilled chicken containing 33 ng/g of PhIP (a portion of the entrée was reserved and later analyzed using previously published methods [19]). Urine was collected for approximately 4 h, 4–8 h after eating the meal.

2.5. Extraction of PhIP metabolites

Urine samples (5 ml) were spiked with internal standard (4.2 ng, in 5 μl water) and applied to a pre-conditioned 60 mg Oasis SPE macroporous polymeric column (Waters, Milford, MA, USA). Metabolites were eluted with 5 ml methanol. The elution aliquot was evaporated to dryness under nitrogen and the metabolites were re-dissolved in 2.5 ml 0.01 *M* HCl. Proteins and high-molecular-mass contaminants were removed by filtering the solution through a Centricon YM-3 centrifugal filter (Millipore, Bedford, MA, USA). The samples were centrifuged in the filter at 3000 *g*, overnight. The filtrate was applied to a pre-conditioned benzenesulfonic acid column (SCX, 500 mg, Varian, Harbor City, CA, USA) and the column washed with 6 ml of 10% (v/v) methanol in 0.01 *M* aqueous HCl. The metabolites were eluted onto a coupled C_{18} column (Bakerbond spe, 1000 mg, J.T. Baker, Phillipsburg, NJ, USA) with 0.05 *M* ammonium acetate, pH 8. The C_{18} column was washed with 3 ml of methanol–water (5:95, v/v) and eluted from the C_{18} column with methanol–water (50:50, v/v). The metabolites were dried under nitrogen and 1 ml urine equivalent was injected into the LC–MS–MS in a volume of 20 μl.

Chromatography was done on a Microtech Ultra-Plus HPLC system (Sunnyvale, CA, USA) equipped with a YMC ODS-A column (250×3.0 mm). Metabolites were eluted at a flow-rate of 200 μl/min using a mobile phase of A (water–methanol–acetic acid, 97:2:1) and 5% B (methanol–water–acetic acid, 95:4:1) for 1 min, to 25% B at 5 min, and a linear gradient to 100% B at 30 min and held for 5 min.

Analytes were detected with an ion trap mass spectrometer (model LCQ, Finnigan, San Jose, CA, USA) in the MS–MS positive ion mode using an electrospray interface. The capillary temperature was 240°C and the spray voltage was 4.5 kV. The sheath gas was set at 70 units and no auxiliary gases were used. The ion trap injection time was 1000 ms and a setting of one microscan was used.

Alternating scans were used to isolate $[M+H]^+$ ions at mass 417, 401, and 321 for natural PhIP metabolites, and 422, for the pentadeutero-labeled internal standard metabolite. Collision energy was 25%. Daughter ions were detected at appropriate masses: 241 $[M+H\text{-glucuronic acid}]^+$ and 225 $[M+H\text{-glucuronic acid-OH}]^+$ from 417 for the *N*-hydroxy-N^2 and *N*3 glucuronide, respectively, 225 $[M+H\text{-glucuronic acid}]^+$ from 401 for the PhIP N^2 glucuronide, 241 $[M+H\text{-}SO_3]^+$ from 321 for PhIP-4′-sulfate, and 246 $[M+H\text{-glucuronic acid}]^+$ and 230 $[M+H\text{-glucuronic acid-OH}]^+$ from 422 for the internal standard, *N*-OH-[$^2H_5$-phenyl]PhIP-N^2-glucuronide. An external standard of naringenin was used in later samples, its $[M+H]^+$ ion isolated at mass 273 with protonated fragments detected at mass 147, 153, and 185.

2.6. Recovery studies and precision of the assay

The overall recovery of the metabolites was determined by spiking each urine sample with known amounts of *N*-OH-[$^2H_5$]PhIP-N^2-glucuronide. Final metabolite amounts were adjusted based on the recovery of the internal standard. The effect of the urine matrix on the recovery of the metabolites was determined by spiking increasing amounts of the internal standard in 5 ml of water and comparing these recoveries to the recovery of the internal standard in 5 ml urine.

Ion suppression in the mass spectrometer by co-eluting interferences was investigated by spiking human urine extracts with mouse urine containing high levels of metabolites. In our method, the *N*-OH-[$^2H_5$-phenyl]PhIP-N^2-glucuronide is used as a surrogate standard for all of the metabolites because of the structural similarity of the metabolites and our belief that it is representative of the other metabolites, within the precision of other aspects of our assay. An external standard of naringenin added to later samples shows that ion suppression is consistent and suppresses the signal by 65% compared to the external standard injected alone.

Replicate analyses of several different urine samples were made during the course of the study to determine the precision of the assay. The coefficient of variation was approximately 28% for urine extractions and LC–MS–MS, with much of the variation occurring in the LC–MS–MS instrument. Consequently, samples were injected three times and the results averaged.

3. Results and discussion

3.1. Method development and urine analysis

The goal of this work was to develop a method that reliably quantifies PhIP metabolites and could be applied to large numbers of urine samples. The initial step of the method utilizes non-specific adsorption to remove all the metabolites from the water and salts in the urine. Other materials were tried in preliminary work, such as C_4, C_8, and C_{18} solid-phase extraction materials and styrenedivinylbenzene medium packed into columns, but none recovered all four metabolites as well as the polymeric material in the Oasis columns.

Our initial attempts at sample clean-up resulted in samples that did not chromatograph well. Poor HPLC column lifetime, peak broadening, and increasing retention time for two of the metabolites, N^2-OH-PhIP-N^2-glucuronide and PhIP-N^2-glucuronide were the symptoms of this problem. Suspecting that urinary proteins and larger molecule contaminants were the cause of some of these symptoms, they were removed by centrifuging the extracts through a filter with a molecular mass cut-off of 3×10^6. Protein determinations of the urine samples before and after filtering demonstrated that 60–80% of the color-reacting material could be removed from the sample during the filtering step (data not shown). This improved HPLC column lifetimes somewhat. After the centrifugation step, further purifications exploited the protonation of the heterocyclic nitrogen atoms that are common to the all the metabolites. This ion-exchange adsorption step was designed to remove uncharged interferences. Finally, the urine extract was concentrated and washed on reversed-phase silica.

To monitor the recovery of the metabolites through the method, a deuterium-labeled internal standard is added to the urine before extraction. Typical recoveries range from 37 to 40%. Final metabolite levels for each sample were adjusted based upon the recovery of the internal standard in that sample. Because of the small peak sizes in the assay, there is variation inherent in the mass spectrometry detection. To account for this variation, each urine extract was injected three times and the peak areas averaged. Variation within samples ranged from 20 to 30%.

Because of the complexity of the urine extracts and the low amounts of metabolite present, metabolites could not be seen by UV or fluorescence detection. Mass spectrometry must be employed.

Urine samples from rodents receiving high doses of PhIP were used to optimize the HPLC separation and the fragmentation of the metabolites. Metabolites in rodent urine were used to determine the linear range of the instrument. The LC–MS–MS peak areas were linear over the range of peaks seen in this study, which is approximately 20-fold higher than the limit of detection. Internal calibration curves

were calculated for each metabolite based upon rodent urine spiked into a human urine matrix. R^2 values were: N^2-OH-PhIP-N^2-glucuronide, 0.9703, PhIP-N^2-glucuronide, 0.978, PhIP-4′-sulfate, 0.999, and N^2-OH-PhIP-N^3-glucuronide, 0.9954.

Further, because of the co-elution of hundreds of compounds into the mass spectrometer, no signal can be seen above the background with single ion monitoring MS for the parent masses (Fig. 1A). MS–MS detection is necessary for these analyses. Fig. 1B shows a human urine sample analyzed by LC–MS–MS, showing peaks for the fragments of four metabolites after the isolation of the parent masses.

Volunteers are asked to refrain from eating meat for 24 h before eating the cooked chicken, and a control urine sample is collected at the end of the meat-free period. A chromatogram that represents a typical sample of control urine is provided in Fig. 2A. No metabolite peaks are seen at the retention times of PhIP metabolites. Fig. 2B represents urine from the same individual, collected during the first 6 h after consuming the chicken. Peaks are clearly seen for each of the four PhIP metabolites.

Fig. 3 shows the percentage that each individual metabolite represents of the total of all metabolites excreted over 24 h for 10 individuals. The N^2-OH-PhIP-N^2-glucuronide was the major metabolite in all cases. PhIP-N^2-glucuronide is the second most abundant, but the ratio of these two metabolites varies from almost equal amounts for subject 2 to 9-fold more N^2-OH-PhIP-N^2-glucuronide in subject 6. With the exception of subject number 10, N^2-OH-PhIP-N^2-glucuronide and PhIP-N^2-glucuronide together account for 90% or greater of the total metabolite excreted. Subject 10 excreted a much higher proportional amount of N^2-OH-PhIP-$N3$-glucuronide (22%) in contrast to the other individuals, in whom N^2-OH-PhIP-$N3$ glucuronide accounted for 7% or less of the total metabolite excreted. The time of excretion of metabolites also varies (data not shown), with some individuals excreting most of the metabolites in the 0–6 h time period and some later, in the 6–12 h time period. Little or no metabolite is detected in the 18–24 h time period.

To extend our method to real-life exposures, we collected urine from an individual who had consumed chicken as part of a restaurant meal. Fig. 4 shows the LC–MS–MS chromatogram of a urine extract collected 4–8 h after consuming the meal. Peaks for all four metabolites and the deuterium-labeled internal standard can be detected.

Our method provides an opportunity to study a genotoxic dietary carcinogen at realistic levels in humans. PhIP is of special interest because it causes tumors in animals that are among the most common cancer sites in humans: the breast, colon, and prostate gland. In addition, exposure to PhIP need not be ubiquitous, but can be determined and modified through intervention, making PhIP-induced tumor formation preventable.

Several different types of studies can be supported by this analysis method. Relative amounts of PhIP metabolites can be used to determine individual metabolic phenotype. The effect of diet on carcinogen metabolism can be determined by controlled feeding studies that analyze the changes in the relative amounts and time of excretion of metabolites. Urine metabolites can also be quantified for individuals on a normal diet, to monitor for exposure levels.

The enzymes known to be involved in the metabolism of PhIP are found at varying levels and activities within the human population [20]. The expression of specific activating enzymes has a great affect on the biological reactivity of PhIP. We believe that the N^2-OH-PhIP-N^2-glucuronide and N^2-OH-PhIP-$N3$-glucuronide metabolites represent the metabolic products of activation pathways, whereas the PhIP-N^2-glucuronide and 4′-PhIP-sulfate represent detoxification pathways. The variation that we detect in these metabolites suggests that the levels of both activation and detoxification enzymes varies among individual volunteers and may be a way to quantify individual phenotype or genotype. Using our method to generate a metabolic profile could provide an indication of potential susceptibility to DNA damage, mutation, and cancer.

On possible mechanism for the protective effects of fruits and vegetables seen in human cancer studies is the influence of natural compounds on both primary and secondary metabolism. This suggests that the metabolism of carcinogens, including PhIP, can be modified by the addition of protective foods to the diet. Our method provides an invaluable tool for monitoring the effect dietary interactions on PhIP

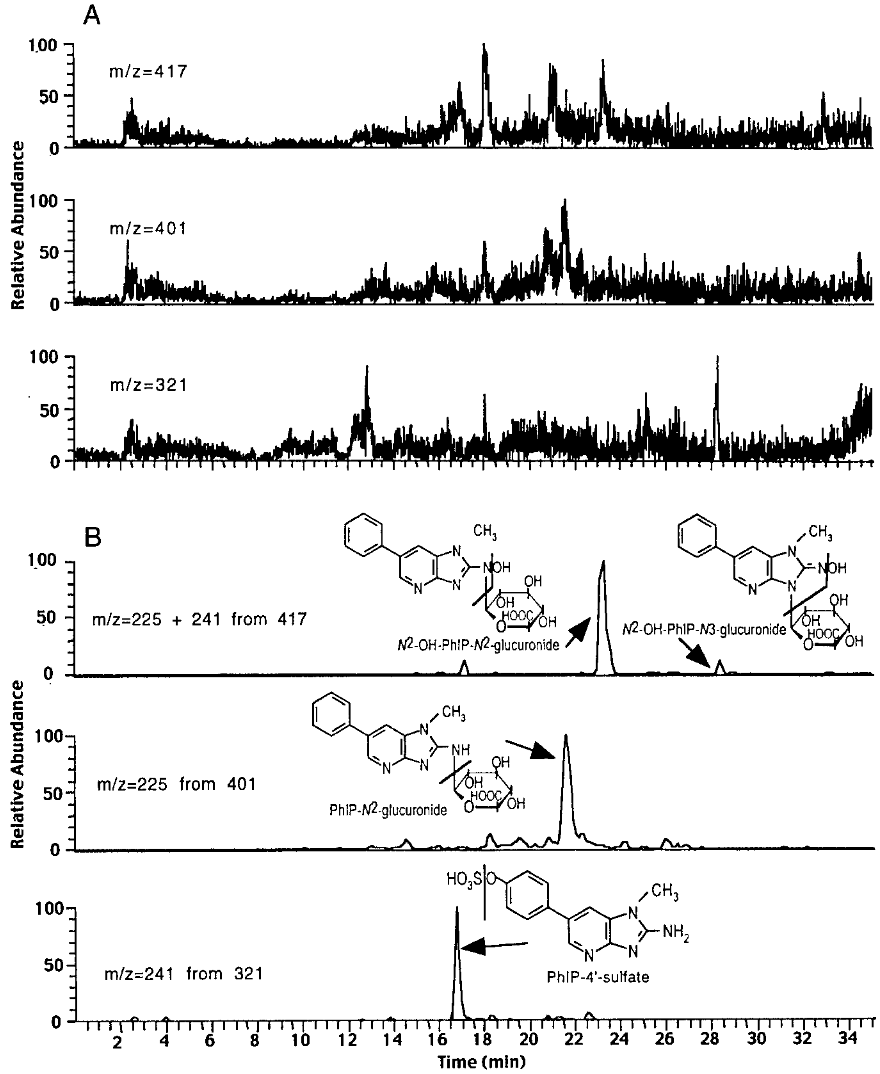

Fig. 1. Reversed-phase HPLC mass chromatograms of urine extract. (A) shows full scan plot of the m/z 417, 401 and 321 corresponding to PhIP metabolites. (B) MS–MS chromatograms of the human urine sample with masses isolated as indicated. Peaks are clearly seen for four metabolites indicated by arrows. Chemical structures and a line indicating the site of fragmentation for each structure are shown.

metabolism. These effects on metabolism can be quantified in humans at normal dietary levels using our method.

Determining the dietary dose of PhIP is important for epidemiology studies and risk determination. Typically, exposure estimations are made through dietary questionnaires. However, the formation of PhIP is variable, and the amount in foods depends on the cooking methods. Dietary surveys have several flaws, including bias, inconsistent reporting, and most importantly, the difficulty in quantifying cooking doneness via questionnaire. As a result, dietary surveys give varying estimates of PhIP amounts that may or may not reflect actual exposures. PhIP metabolite detection in the urine of the subject who ate chicken prepared at a restaurant demonstrates that our method is sensitive enough to monitor PhIP exposure of individuals in real-life situations.

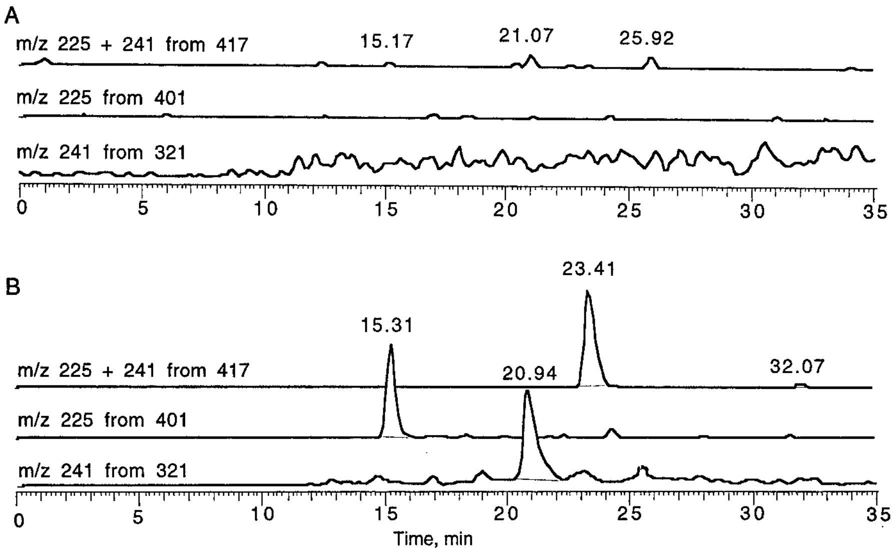

Fig. 2. LC–MS–MS chromatograms of urine from a subject abstaining from well-done meat for 24 h (A), and urine collected 0–6 h after consumption of well-done chicken (B).

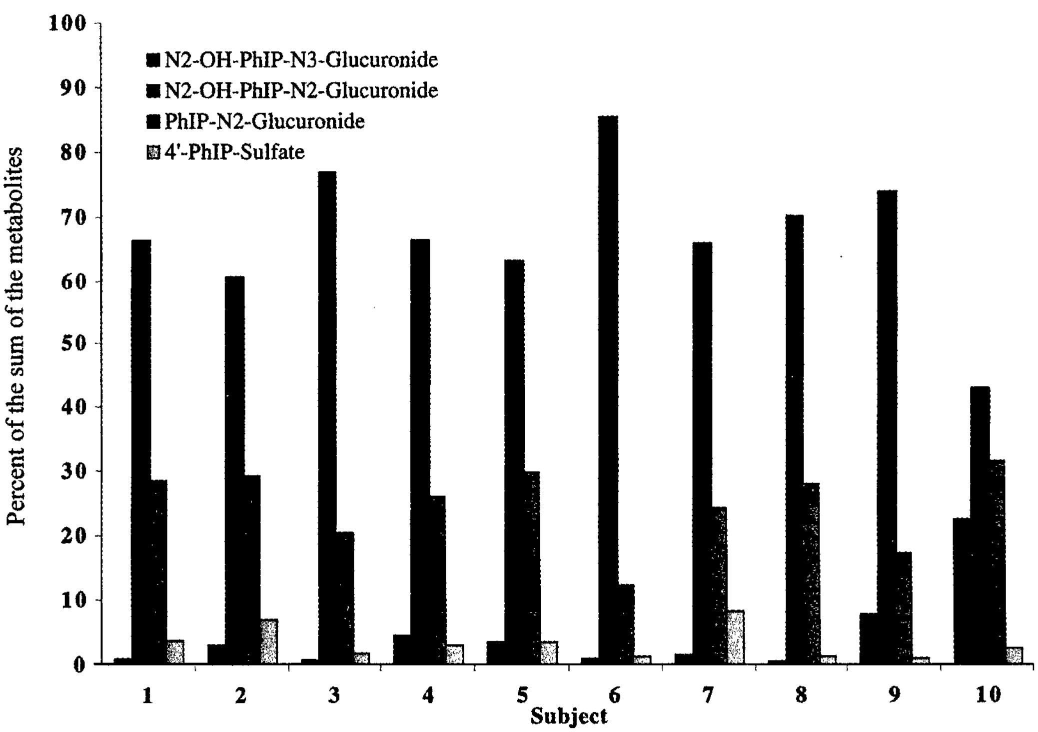

Fig. 3. Graph of individual PhIP metabolites excreted over 24 h from 10 individuals eating a single meal of well-done chicken.

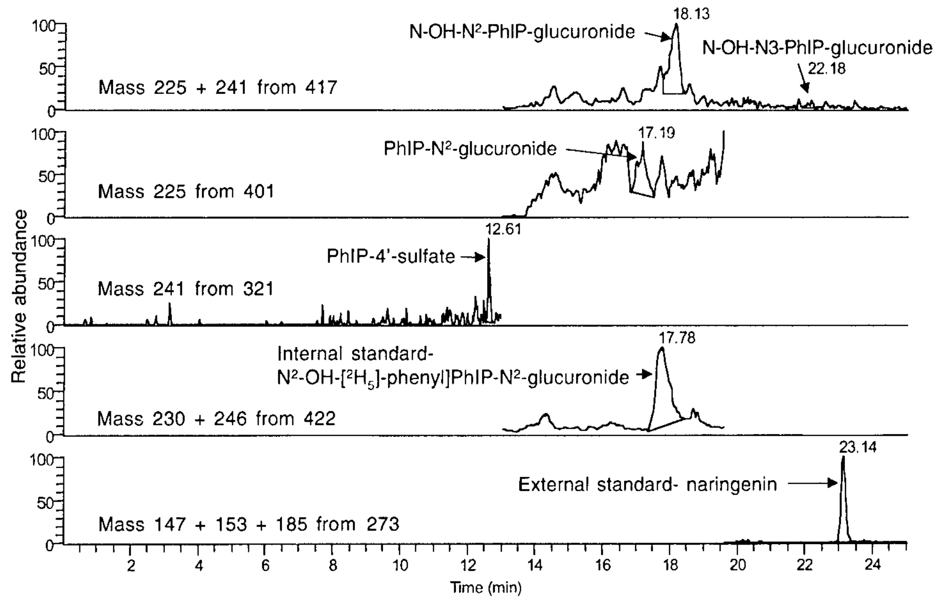

Fig. 4. LC–MS–MS mass chromatograms of urine collected after consumption of a restaurant meal of grilled chicken. Peaks identified are at the retention time of metabolites or the added internal or external standard. The equivalent of 2 ml of urine and 5 ng of internal standard were injected.

Future studies will focus on improving the method by increasing the sensitivity of metabolite detection, allowing us to lower the amount of food containing PhIP given to the volunteers. Reducing the analysis time and variation for the LC–MS–MS analysis are also needed. Repeated analysis of PhIP metabolism in the same individuals over time will help determine the consistency of PhIP metabolism, allowing us to correlate the PhIP metabolite phenotype with genotype.

Altering the metabolism of PhIP to prevent formation of biologically active species may reduce individual susceptibility and prevent the occurrence of cancers in target tissues. The method described here should make studies of individual susceptibility and dietary interventions possible in the future.

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